CYCLIN D1 EXPRESSION IN LARYNGEAL CANCER

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CHAPTER 1

INTRODUCTION

Laryngeal squamous-cell carcinomas (LSCC) comprise the vast majority (96%) of laryngeal malignancies [1]. Larynx has several clinical and molecular peculiarities. The American Cancer Society classifies the larynx as part of the respiratory system, separate from the oral cavity and pharynx [2]

Most of these tumors originate in the glottis and supra glottis; the sub glottis is an extremely rare site of origin. The estimated incidence of cervical lymph-node metastases with no obvious primary site (occult T) is from 3% to 9% 5, 6 and some of these might reasonably have a laryngeal (especially supra glottic) origin. The male/female ratio for the incidence of laryngeal cancer is much higher than in other parts of the head and neck [2].

Differences in chromosomal pattern and carcinogenic progression between LSCC and other head-and-neck squamous-cell carcinomas (HNSCC) have been detected by comparative genomic studies [3].

Practically all patients (95%) with LSCC have a history of tobacco smoking, which increases risk in a dose-dependent way.

The standard options for treatment of LSCC are surgery, radiotherapy, chemotherapy, or a combination of these. Radiotherapy is used in more than 70% of patients, surgery in about 55% and chemotherapy in about 10%. It is widely accepted that most early-stage LSCC can be adequately treated with single-modality therapy, whether surgery or irradiation, with a 5-year local control of 85–98% [10, 13, 14].

A multimodality approach based upon a combination of surgery and irradiation is the most common treatment for stage III and IV Disease [1].

The increasing use of chemo, radiotherapy and more conservative surgery to preserve organs and their function [1] may have led to a better quality of life for LSCC patients, but has clearly failed to improve survival, which remains the primary aim.

Five year survival rates for cancer of the larynx did not show any improvement over the last 30 years [2]. In LSCC, we can identify several potential reasons for this failure.

The majority of patients with LSCC (more than 60%), especially glottic cancers, present with early stage disease, and early diagnosis remains the best

predictor for survival. There is a reported increase in patients diagnosed with stage IV cancers, particularly in the supra glottis. Less than 1% of patients with LSCC are asymptomatic at presentation [1].

As the sensitivity of the most frequently used diagnostic procedures, though relatively high, is not absolute 97% for direct laryngoscopy, 90% for indirect laryngoscopy, 80% for CT of the primary site [1], a number of LSCC patients may escape the first diagnostic approach. For most of its natural course, LSCC is clinically silent and even histologically occult, so no current protocols for clinical screening can sufficiently anticipate tumor detection.

The clinical TNM often underestimates the extension of the disease when compared with the real pathological TNM, which places a significantly higher proportion of tumors in the advanced-stage (III or IV) group [1]. Clinical methods are inaccurate predictors of pathological findings and may underestimate disease extension and macroscopically uncertain margins.

Therefore, the TNM cannot be adequately evaluated in patients treated exclusively by radiotherapy, in the absence of a surgical specimen. After irradiation, it can become very difficult to assess data obtained by imaging and endoscopy for the diagnosis of both minimal residual disease and early recurrence.

Despite the multiplicity of clinical prognosticators, the only consistent clinical predictors for disease control and disease-specific survival in LSCC are T and, to a greater extent, N. [7,8,11].

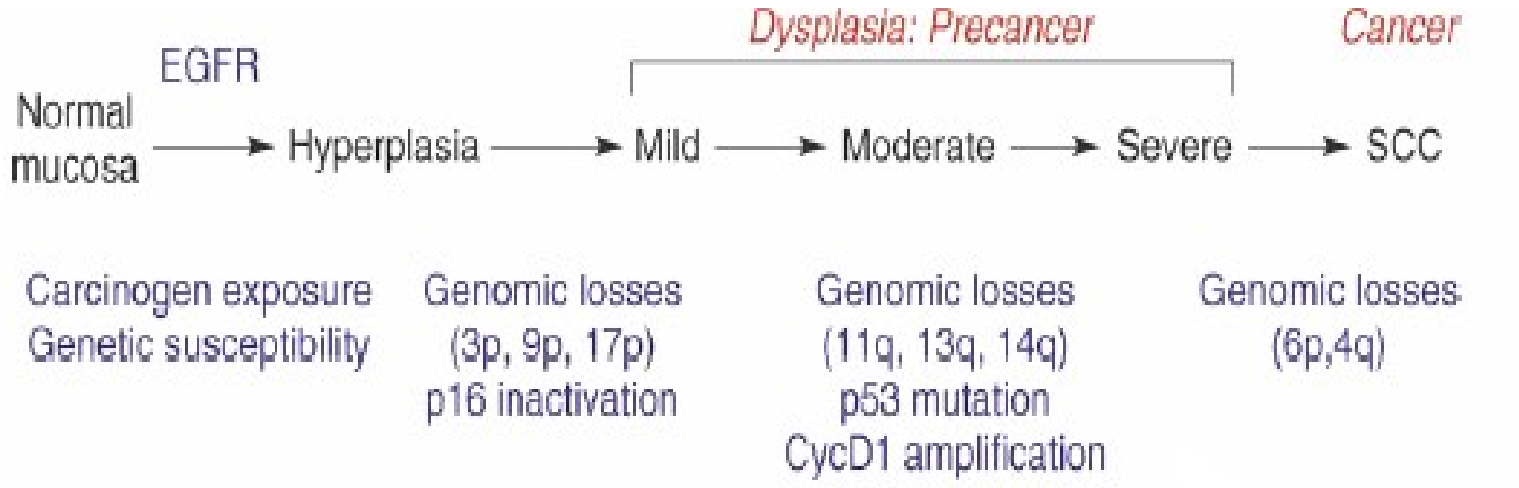
The prognostic stratification of LSCC patients is inadequate since similar patients, affected by tumors with similar clinico pathological features and undergoing the same treatment, may differ widely in prognosis, probably due to the extreme biological heterogeneity of LSCC, which contributes to the lack of consistency in treatment planning.

So a different approach to LSCC, based on genetics and molecular biology in addition to the clinical and histological approach, is required to overcome these obstacles and to reduce cancer-related mortality in LSCC patients.

1.1 Potential clinical application of molecular markers

Although the best-known risk factors, clinical TNM and histopathological grading will retain their value, it is now possible to acquire biological information about host and tumor to optimize the management of LSCC.

Systematic study of biological markers might be integrated into clinical practice in the phases of prevention as ‘molecular epidemiology’ of diagnosis as ‘molecular diagnostics’, of prognostic assessment and treatment selection as ‘molecular characterization’ [12] , and of the synthesis of new drugs as ‘molecular targeting’.



ic alterations currently
ion in relationship to

receptor; p16, p16
SCC, squamous cell

1.2 Molecular epidemiology

The best-established risk factors for LSCC are behavioral, e.g. cigarette smoking and heavy drinking. Primary prevention can be easily obtained by abandoning adverse habits, but not all LSCC patients have a history of behavioral risk factors or clinically evident precancerous lesions.

Molecular epidemiology should help us to recognize patients and/or areas of laryngeal mucosa with a high susceptibility for developing LSCC, and possibly to identify molecular targets for effective secondary prevention (chemoprevention).

The incidence of genetic alterations in dysplastic, pre malignant lesions is greater than half that found in invasive HNSCC [13]. The latency between carcinogen exposure and the appearance of malignancy may be as long as 25 years, so important molecular alterations should be detectable in affected mucosa many years before an invasive phenotype is produced, and presumably some of these will be more strongly associated with progression toward carcinoma.

Several cellular alterations have been tested in clinical studies as potential markers of commitment to transformation (molecular histopathology), both in pre malignant lesions and in apparently healthy mucosa [14-17]. Markers of commitment could help in the early diagnosis of malignant transformation by stringent follow up of high-risk mucosal areas and in timely secondary prevention by immediately evaluating its effects at the molecular level [15].

The p53 pathway is of great interest in this aim. p53 is normally expressed in LSCC more frequently and the p53 gene has a mutation pattern more similar to that in lung SCC than in other HNSCC [4] .Alterations in p53 status have been extensively studied in tumor cells, in precancerous lesions and in apparently healthy mucosa of HNSCC patients, in the hope of verifying the intriguing hypothesis that they might predict the development of SCC [18, 18-20]. Mutations of p53 have also been evaluated to establish whether multiple primary tumors have a mono- or polyclonal origin[21, 22].

Although no definitive conclusions have yet been drawn about these fundamental issues, a coherent model is now beginning to emerge [23] and the use of p53 alterations as a marker to identify ‘condemned mucosa’ remains an intriguing, if still hypothetical, possibility.

In LSCC, p53 expression is altered less frequently, with a mutation pattern different from that of other HNSCCs (and more similar to lung SCC) [4]. If we assume that the p53 pathway is impaired in some way in every malignant epithelial neoplasm, then alternative mechanisms of inactivation could be particularly relevant in LSCC [4].

Degradation mediated by other cellular proteins, such as mdm2 [24], or by human papilloma virus (HPV) E6 oncoprotein [25], may represent two such alternative pathways leading to loss of p53 function. Chromosomal alterations, such as 9 p21 loss, are prevalent and early events in carcinogenesis, and proposed targets for preventive strategies [13, 15].

The over expression of the epidermal growth factor receptor (EGFR) [14], alterations in Cyclin D1 (in the earliest phases, over expression, and later CCND1 amplification) [14, 26] and high telomerase activity [27] are early events, and may be potential markers for the prediction of neoplastic progression. Individual susceptibility to LSCC may derive from environmental or genetic factors. A genetic predisposition to the development of LSCC is highly probable.

Intrinsic sensitivity of cells to mutagens such as bleomycin is a biomarker of HNSCC susceptibility [28]. Polymorphisms of carcinogen, metabolising enzymes known to be involved in the metabolism of carcinogens found in tobacco smoke are relatively common in most populations.

A growing body of evidence suggests that many of these genetic polymorphisms are associated with the risk of developing cancers of the aero digestive tract [29-31].

In particular, the risk of developing LSCC has been evaluated in relation to polymorphisms of genes encoding for aryl amine N-acetyl transferases [32], human OGG1 DNA repair enzyme [33], CYP1A1, XRCC [31] and glutathione S-transferases, with controversial results [35, 36].

These and other detoxifying enzyme genes might be evaluated in the future to assess susceptibility to environmental carcinogens and thus the risk of developing LSCC in association with tobacco smoking.

1.3 Molecular diagnostics

Molecular diagnostics should help us to:

1. Diagnose biological transformation, even with negative histology;
2. Detect extremely early neck node involvement (occult metastases);
3. Assess precisely, even in the absence of surgical specimens, the local and regional spread of the tumor;
4. Detect minimal residual disease at the margins of surgical resection and at the primary site after irradiation;
5. Diagnose extremely early recurrences.

The clinical goals of molecular diagnostics are earlier, initial or salvage treatment and safer oncological effectiveness with better functional results. The detection of minimal residual disease and of early recurrences and metastases by molecular markers would be particularly useful after radiotherapy, when for several months radiation damage makes the evaluation of treated sites more difficult, often with fatal diagnostic delay.

Histopathologically benign mucosa of the upper aero digestive tract may harbor foci of clonal, preneoplastic cells that are the site of origin of genetically related, metastatic HNSCC. The use of multiple biopsies aiming to map, for example by micro satellite analysis, the mucosal sites that harbor these clones may be a useful tool [6].

The perfect marker for molecular diagnostics should be present in all LSCCs, easily detectable even in histologically occult cases (high sensitivity), indicative of non-reversible passage from severe dysplasia to cancer (capable of differentiating precancerous lesions from early carcinomas) and thus always absent in non-cancerous mucosa (high specificity).No biological marker with these characteristics has yet been described; all molecular features proposed so far only approximate such an ideal.

One of these is eIF4E over expression, which can be demonstrated in practically all LSCCs and whose presence in histologically tumor-free surgical margins predicts recurrence with discrete specificity and good sensitivity [68].Loss of p16 expression is one of the most frequent molecular abnormalities in HNSCC [69] and seems be important in laryngeal carcinogenesis [70, 71], but its clinical potential in LSCC requires further evaluation. p53 mutations appear to predict local recurrence when detected on surgical resection margins [72].An alternative cytogenetic approach has been proposed by Califano et al. [13].

1.4 Molecular characterization

Molecular characterization by the study of predictive molecular factors aims to define homogeneous groups of patients for prognostic stratification and treatment selection. Although a plethora of studies have sought to evaluate their potential, no molecular marker yet contributes to clinical decision-making.

Attempts have been made to outline how molecular markers might be integrated with standard TNM and histological grading (clinico pathological parameters). The perfect marker for molecular characterization of LSCC should not be constantly present in malignant cells but invariably associated with precise biological features and predictable clinical behavior, and easily detected by a standard, reliable and simple assay on a small sample such as from a biopsy. No such marker yet exists.

Recently, cDNA micro arrays, a powerful tool from which large amounts of genetic information can be obtained, have been used for an initial tentative molecular classification in HNSCC, based on patterns of global gene expression [73,74]. This method can only be applied to frozen tissues, because RNA is destroyed during formalin fixation, and a frozen tumor bank combined with a strong clinical database and complex statistical capacity would be required to make full use of this expensive technology.

Searching for three or four well-defined biological markers with more reliable assays might allow us to classify tumors as positive (Mc+) or negative (Mc-) for molecular characterization. We should try to assess at least some of the main biological features of tumors, such as aggressiveness, invasiveness, radio, and chemo sensitivity. . These characteristics, intrinsic to a given tumor and also some of the molecular markers studied have a large influence on prognosis and should guide therapeutic decisions.

TNM staging could then become TNMMc staging, resulting in better prognostic stratification of patients and the selection of the most suitable, individualized treatment. It would prevent the over treatment of Mc-ve patients and, most importantly, the under treatment of Mc+ patients, which has probably contributed to the cited failure to improve prognosis in LSCC over the last 30 years.

In Mc+ tumors, larger resection margins could be planned with a more ablative approach to primary tumor, since conservative surgery is too risky in these cases. In cN0 patients, a more aggressive approach in the neck would involve elective bilateral dissection or elective irradiation.

Adjuvant radiotherapy could be advised also in tumors with histologically negative resection margins.In Mc –ve tumors; integrated staging might allow safe indications for conservative surgery and thereby functional preservation, lowering at the same time the risk of failures.

1.5 Characterizing molecular markers

Among the markers evaluated so far, some have appeared potentially reliable and suitable from a clinical perspective.

i. Epidermal growth factor receptor (EGFR)

There is strong evidence of a role for EGFR expression (and, to a lesser extent, for its ligand, transforming growth factor-*a*) in predicting prognosis, because it adversely influences overall relapse-free and metastasis-free survival in LSCC.

EGFR retains a strong predictive value independently of treatment (surgery, chemotherapy and radiation) [75-80], which makes it, the most reliable prognostic molecular marker at present. Furthermore, EGFR over expression seems to predict both chemo- and radio resistance [79, 81].

It is a receptor for growth factors (as TGF and EGF) with tyrosine kinase activity in normal cells. Upstream activator of MAP kinase pathway and of other pathways involved in cell growth, cell migration, block of apoptosis. It is frequently and early over expressed in LSCC, mainly by post-translational mechanisms. At present the most reliable biological marker for molecular characterization, marker of aggressiveness [75, 76] and of invasiveness [77].

ii. p53

Alterations of p53 protein expression and mutations of the p53 gene have been extensively studied as predictors; changes in p53 are proposed independent predictors of recurrence in LSCC [24,82], but this prognostic value is controversial[4], especially in surgically treated patients [83].

p53 over expression, detected by immunohistochemistry (IHC) in an high percentage of LSCC [84], appeared to correlate well with p53 mutation [85],but a recent study has shown significant discrepancies between p53 IHC and genotyping data [86].

p53 gene mutation has been suggested as more reliable than IHC over expression for characterization and reportedly predicts the response to radiotherapy in LSCC patients [86]; this observation is consistent with the biological role of p53, which mediates apoptosis associated with DNA damage.

iii. CYCLIN D 1

A separate prognostic role has been described for Cyclin D1 protein over expression [87] and CCND1 gene amplification [88], with an impact on relapse-free and overall survival in HNSCC. Studies on breast tumors and also on HNSCC have shown that CCND1 amplification, rather than protein over expression, might have prognostic value [119].

iv. Over expression and amplification of Cyclin D1 gene (CCND1)

Cyclin D1 gene transcriptional activity normally strictly depends on mitogen stimulation, and leads to cell commitment to mitosis through START checkpoint. An early CCND1 over expression is often detectable without evidence of gene amplification; it can be used for molecular epidemiology but it seems to retain a lower prognostic value, if compared with CCND1 amplification, a marker of aggressiveness in LSCC [88].

The cyclin D1 gene plays a pivotal role in the regulation of the cell cycle[89]. During the middle to late G1phase[90], it complexes with cdk4 or cdk6, thus promoting the phosphorylation of the pRb gene product and, finally, progression to the S phase[91].

It has been shown that cyclin D1 can become deregulated as a result of various genetic lesions, including chromosomal inversion and translocation in parathyroid adenomas and B-cell non-Hodgkin lymphomas [92, 93] or gene amplification in carcinomas of the breast [94, 95] liver[96] pancreas [97] esophagus [98]anus [98]ovary[99]bladder [100] and lung [101]

Experimental observations indicate that a moderate over expression of Cyclin D 1 leads to a shortening of the G1 phase and less dependence on growth factors for cell proliferation. [102,103] .It has likewise also been recently reported that antisense to Cyclin D 1 is capable of inhibiting the growth and tumorigenicity of human colon cancer cells, which thus suggests that increased cyclin D1 expression might contribute to the abnormal growth of tumor cells[104]

Cyclin Dl gene aberrations are frequently detectable in head and neck tumors[105-110, 88,111-113] especially in carcinomas of the hypopharynx and, to a lesser extent, in those of the oropharynx and larynx[114-116]

It has been reported that Cyclin D 1 amplification and/or over expression are associated with unfavorable clinico pathologic features[114, 116] tumor recurrence, and a poor prognosis[111, 115,117,118]

v. Telomerase activity

In a normal cell a low telomerase activity, required for telomere lengthening and autonomous replication, can be detected in haematopoietic tissue, in some immune cells (activated lymphocytes), in basal epithelial layers. Absent in most non-transformed differentiated cells.

It is present, often at high levels, in most laryngeal cancer cells. It can at least partly depend on h-TERT gene (coding for catalytic subunit of telomerase) over expression [27].

vi. Cathepsin D

It is a Lytic enzyme active in extra cellular matrix rearrangement. An over expression is often detectable in tumor cells, where it seems to contribute to invasiveness [119].

vii. Human papillomavirus (HPV)

Normally absent in cells, HPV affect epithelia with mucosal or epidermal tropism according to genotype. In tumor cells important onco suppressors such as p53 and pRb are inhibited and degraded by HPV oncoproteins. In turn, over expression of Oncogenes such as EGFR and Cyclins A and B is induced [40, 51-53].

viii. Type II oestrogenbinding sites (EBS)

These are normally present in laryngeal mucosa. Type II EBS may at least partly mediate tumor growth inhibition by tamoxifen and quercetin; possible targets for chemoprevention and therapy [122].

ix. S100-A2 Ca2+ binding protein

Increasing levels of expression during differentiation of squamous epithelial cells; absent in basal layers. Under expression in cancer cells, are inversely proportional to tumor differentiation. Starting from data on NSCLC a role as a real oncosuppressor has been hypothesized [121].

x. Methyl-p- hydroxyphenyllactate esterase (MEPHLase) activity

Enzyme involved in the metabolism of methyl-p- hydroxyl phenyl lactate, ligand of type II EBS, with a role in growth and differentiation of several tissues (breast, uterus), normally expressed in larynx. In LSCC a low activity is associated with poor differentiation, and shorter overall survival and metastasis-free survival [123].

xi. Type 2 cyclo-oxygenase (Cox-2)

Enzyme involved in arachidonic acid metabolism and autacoid synthesis, induced by various stimuli in several cell types. Cox-2 activity seems to promote tumor neo angiogenesis. Nevertheless, evidence exists that low Cox-2 expression indicates poor differentiation and higher aggressiveness and invasiveness [61].

xii. Galectin-3

Galectin-3 is a pleiotropic carbohydrate-binding protein participating in a variety of cell processes, and mediating cell-to-cell interactions.Galectin-3 expression seems positively associated with tumor keratinisation and histological grade. A significant correlation was found between galectin-3 tumor positivity and longer metastasis-free and overall survival in LSCC patients [120].

Various problems affect the clinical application of molecular markers for tumor characterization. The perfect marker has yet to be demonstrated; in particular the detection assays must be practical and reliable, and should be widely available.

The inconsistency of assay methods for most factors studied, and patient and treatment heterogeneity, all detract from an ability to draw definitive conclusions. Meta-analysis of published data and by multidisciplinary and multi centre clinical trials is needed to evaluate every molecular marker proposed for clinical practice.

1.6 Objectives of the study

1. To analyze the expression of Cyclin D 1 – a cell cycle regulatory protein, in squamous cell carcinoma of larynx patients

2. To correlate the expression of Cyclin D1 with clinical and pathological parameters like Age of the patient, Extent of tumor and Nodal status, Grade and Stage of the tumor, any predilection for sub sites in the larynx.

3. To investigate whether over expression of Cyclin D 1 is associated with an increased likelihood of tumor recurrence and over expression of Cyclin D 1 could serve to identify a proportionally distinctive group of patients.

1.7 Scope of the study

On the basis of these immuno histochemical results, it was possible to select a subgroup of patients with a high risk of recurrence and poor prognosis to undergo more extended surgical treatment and/or combination anti tumoral therapeutic procedures.

The goal is to block specific pathways involved in the carcinogenic process or in tumor pathogenicity (aggressiveness, invasiveness), and to restore effective oncosuppression and possibly in the treatment (Antisense Cyclin D 1)

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The retrospective study group consisted of **74** patients with squamous cell carcinoma of larynx treated in Cancer Institute (WIA), between January 2000 and December 2003.

These patients were selected randomly based on the inclusion criteria:

1. No history of previous malignancy
2. Primary squamous cell carcinoma of larynx only
3. Not received any treatment before

Samples for histopathological examination were obtained prior to starting the appropriate therapy.

71 patients were male and 3 were females. Clinical staging and identification of the anatomical site of the tumors were based on the International Union Against Cancer (UICC) TNM classification of malignant tumors.

15 of them (20.3%) were supra glottic, 47 (63.5%) were glottic and 12 (16.2%) were transglottic tumors. Grade of differentiation of the tumor were made according to Broder's classification. 7 well differentiated, 25 moderately differentiated and 35 poorly differentiated tumors were studied. For 7 tumors grade was not known.

Depending on the stage of the disease these patients were treated with primary radiotherapy; primary surgery and combination of radiotherapy and surgery.

2.2 Methods

Expression of Cyclin D1 was studied immuno histochemically and the primary antibody used was Monoclonal Mouse Anti human Cyclin D1 clone DCS 6 (DAKO cytomation Denmark)

The samples from all of the cases were routinely fixed in 10% buffered formalin and embedded in paraffin. The paraffin embedded section of 5 micron thick was placed on 3'Amino Propyl Triethoxy Selaine (APES) coated slides.

Positive and negative controls were used. Positive control was taken from a carcinoma larynx specimen found to have expressing Cyclin D 1. Same was used as negative control but Cyclin D 1 antibody was not added.

The slides were treated in Xylene consecutively twice for 8 minutes, to de wax the sections and this was followed by treatment with absolute alcohol consecutively twice for 5 minutes to dehydrate them.

Slides were washed in tap water for 8 to 10 minutes taking care not to disturb the tissue sections and then the slides were placed in 0.3 % hydrogen peroxide for 30 minutes, to quench endogenous peroxidase activity in the tissues

2.2.1 Antigen retrieval

Antigen retrieval is needed for exposure of the epitopes for immune reaction. The slides which were quenched of peroxides were wet autoclaved at 121 degree Celsius in 10 mM citrate buffer at pH 6 for antigen retrieval and no holding time is required for it .The autoclaved slides were cooled and washed with Phosphate buffered saline (PBS) for 5 minutes.

2.2.2 Blocking antibody reaction

The slides were arranged on a moist chamber to prevent drying of the sections. 100 micro liters of 2 % BSA (Bovine Serum Albumin) was added to each section, taking care to cover the whole section and left undisturbed for 30 minutes.

Following gentle tipping of BSA off the slides, 75 microlitre of primary antibody (cyclin D1) was added to each section in 1: 75 dilutions. Care was taken to omit the negative control.

The slides were left overnight undisturbed to incubate with primary antibody at 4 – 8 degree Celsius in a refrigerator and on the next day, the slides were washed with PBS thrice for 5 minutes each, to wash off the excess of primary antibody.

2.2.3 Secondary antibody

Secondary antibody (Rabbit anti mouse) was added in 1: 300 dilutions to the slides and incubated for 35 minutes in room temperature. The slides were washed in PBS thrice for 5 minutes each and then ABC (Avidin Biotin Complex) 75 micro litre was added to each slide and incubated for 30 minutes. Throughout the procedure care was taken not to dry up the slides completely.

2.2.4 Treatment with chromogen

Slides were washed in PBS thrice for 5 minutes each, to wash off the excess of ABC and were treated in a solution of DAB (Di Amino benzidine + 150 ml of water + 150 ml PBS + 100 µl of Hydrogen peroxide) for 5 minutes. The excess of DAB was washed off the slides in tap water for 5 to 10 minutes and counterstained in hematoxylene for 2 minutes.

The slides were washed for 5 to 10 minutes in water and fixed in lithium carbonate saturated solution and washed under tap water once again for 5 minutes. The slides were drained well and treated with absolute alcohol twice for 3 minutes each followed by treatment in Xylene twice for 5 minutes each. Then sections were mounted using DPX.

2.2.5 Pathological analysis of expression of Cyclin D 1

Immuno histochemical staining was scored by a pathologist who is not aware of the clinical details. Whole of the microscopic fields of the sections stained were studied for scoring with 40 X magnification.

Scores were ranked as, [87,107,181]

- Negative;

+/- 0-5% tumor cells were positive;

+ 5-50% tumor cells were positive;

++ >50% tumor cells were positive;

Only nuclear staining was taken into account

2.3 Statistical Analysis

Descriptive statistics were calculated with their 95% confidence intervals. Associations between clinico pathologic and immuno histochemical results were evaluated by means of 'Chi Square Test'.

For the purpose of statistical analysis Cyclin D1 was considered in two classes: Positive (> 5% tumor cells were positive for Cyclin D 1 expression) and Negative (No expression of Cyclin D 1 or < 5% tumor cells were positive for Cyclin D 1).

Other investigated prognostic factors were classified as follows—
Age at diagnosis: <30 years, 31-40, 41-50, 51-60, 61-70, 71-80; Histological grade: G1, G2, G3and Gx ; Lymph node metastasis: Positive and Negative; Tumor extension T1a, T1b, T2-4, Clinical stages: Stage I and II together and Stage III and IV together.

Finally distribution of Mean and standard Deviation of time to recurrence and Cyclin D 1 expression ; distribution of Mean and standard Deviation of time to residual disease after treatment with Radiotherapy and Cyclin D 1 expression was studied.

CHAPTER 3

REVIEW OF LITREATURE

3.1 Laryngeal cancer

3.1.1 Epidemiology

Cancer of the larynx is one of the most common cancers seen among male. It ranked within the top ten cancers in males and accounts for approximately 4.1% of all male cancers according to our hospital based cancer registry (1998-2002)[124]**.** The larynx is the second most common site of cancer in the upper aero digestive tract, preceded in frequency only by the oral cavity.

It is one of the few cancers, which has a high male predominance. The average number of cases per year is about 9 fold higher among males in the ratio of 108 females to 1000 males, but recent data show that the ratio of affected males to females is decreasing as the result of an increasing incidence among women[2].

Squamous cell carcinoma accounts for greater than 95% of laryngeal cancers [124]. Glottic and supraglottic larynx are the common sub sites in laryngeal cancer. Women are more likely to develop supraglottic cancer than glottic[125].

The distribution by subsite revelaed that a majority of cases had a Glottic involvement (41%) followed by Supraglottis(27%). 27% of cases not categorized by subsite[126].

At cancer institute the tumor stage data among all cases revealed the following: Stage I (25%), stage II (15%), stage III (33%) and stage IV (23%); stage unknown was 4 %. A slightly less than half of the total cases registered at Cancer Institute received cancer directed treated with the radiation therapy, either alone or in combination with other modalities, forming the majority[126]

Cancer of the larynx is a disease of the elderly, with the peak incidence in the sixth and seventh decades [124,127]. Less than 1% occurs below the age of 30 years [124, 128]. No racial predominance has been demonstrated.

The estimated 5 year survival for cancer of the larynx as a whole, including all stages is 68% making it one of the more curable cancers of the upper aero digestive tract. However this numbers has not changed significantly over, at least, the last 20 years[129]. According to our institute data the overall and disease free survival from laryngeal cancers treated in 1997 and followed through 2002 were 52% and 50% respectively.[126]

In Europe laryngeal squamous cell carcinoma is one of the most frequent malignancies of Head and Neck region, accounting for about 52,000 new cases per year[130]. The yearly incidence rate for men in Northern Europe is approximately 6 per 100,000, rising until 18 per 100,000 in Southern countries[131].

In the United States laryngeal squamous cell carcinoma is estimated to account for almost 0.8 % of all new cases of malignancy, with an incidence of 10,000 cases per year, and to have caused 0.6% of all cancer deaths in 2004[2]. Most of these tumors originate in the glottis or supra glottis; the sub glottis is an extremely rare site of origin[132].

3.1.6 Etiology

As with most tumors, multiple factors contribute to the development of cancer of the larynx. Foremost among them is tobacco. Second is the synergistic effect produced when tobacco is combined with heavy alcohol intake.

Risks were also shown to decline markedly with risk returning to nearly that of non smokers by 15 years after cessation[133]. Alcohol is an independent risk factor only for cancers of the supraglottic larynx[134].

Certain occupations and exposures also pose a higher risk for subsequent cancer of the larynx. Painters, metal-working and plastic-working machine operators, construction workers and those exposed to diesel and gasoline fumes[135, 136] as well as those exposed to therapeutic does of radiation [137].

Dietary factors – A protective effect has been demonstrated for fruits and dark green vegetables possibly due to their high concentration of carotenoids[138]. Salt preserved meats and high dietary fats have been implicated in increasing the risk for cancer of the larynx[139].

The incidence of genetic alterations in dysplastic, pre malignant lesions is greater than half that found in invasive Head and Neck Squamous cell carcinoma[13.] The latency between carcinogen and the appearance of malignancy may be as long as 25 years, so important molecular alterations should be detectable in affected mucosa many years before an invasive phenotype is produced and presumably some of these will be more strongly associated with progression toward carcinoma[14].

The p53 pathway is of great interest in this aim. Alterations in p53 status have been extensively studied in tumor cells, in precancerous lesion and in apparently healthy mucosa of HNSCC patients, in the hope of verifying the intriguing hypothesis that they might predict the development of SCC.

Use of p53 alterations as a marker to identify condemned mucosa remains as intriguing, if still hypothetical possibility[18, 140]. In laryngeal cancer, p53 expression is altered less frequently, with a mutation pattern different from that of others HNSCCs (and more similar to lung SCC) [141].

Chromosomal alterations, such as 9p21 loss, are prevalent and early events in carcinogenesis, and proposed targets for preventive strategies[26]. The over expression of epidermal growth factor receptor (EGFR) [14], alteration in Cyclin D 1(in the earliest phases,over expression and later CCND1 amplification) [142] and high telomerase activity are early events any may be potential markers for the prediction of neoplastic progression.

3.1.7 Diagnosis

The three modalities used to evaluate cancer of the larynx, as with all upper aero digestive lesions, are history and physical examination, radiography and endoscopy.

A history of gradual development of hoarseness, sore throat, dysphagia and odynophagia are common presenting symptoms. Hoarseness is produced early in cancer of the glottis but is a late finding in carcinomas of the supraglottic and subglottic larynx.

Video laryngoscopy allows documentation of the size and extent of the cancer of cancer at presentation and assists with planning operative approaches, especially when conservation laryngeal surgery is anticipated.

In cancers of the supraglottis, involvement of the pre-epiglottic space, paraglottic space, pyriform sinus or arytenoids will often have a major impact on treatment. For cancers of the glottis, involvement of anterior commisure, ventricle, paraglottic space or subglottic larynx will have a similar impact on the treatment chosen.

For cancers of the subglottis, the extent of tracheal and esophageal involvement must be known in order to prepare for appropriate surgical reconstruction or adequate radiation portals. It is in the assessment of these key areas, often hidden from physical examination, that radiography and operative endoscopy are invaluable.[143]

3.1.8 Prognosis

Multiple studies have shown that increasing T stage and increasing N stage correlate independently with lower survival, and that for cancer of the larynx, N stage is more predictive of overall survival than T stage[144].

Extra capsular spread outside the involved lymph nodes also portends a poor prognosis[145]. Overall low patient performance status and presence of nodal metastasis in the inferior aspect of neck (level IV) [146] are other clinical factors associated with a decreased survival from cancer of the larynx.

Tracheostomy prior to definitive treatment of an obstructing laryngeal cancer has been shown in several studies to have an increased incidence of stomal recurrence and decreased overall survival[147, 148]. Peri neural and perivascular invasion both indicate aggressive cancer behavior. The influence of histologic grade of the primary cancer is not certain.

Squamous cells carcinomas are graded into poorly, moderately and well differentiated neoplasms based on their keratinization and nuclear atypia[9]. Generally, well differentiated carcinoma grows in a bulky rather than infiltrative pattern and remains localized for longer periods of time than poorly differentiated cancer.

3.1.9 Treatment

The treatment of cancer of the larynx has evolved significantly over the last two decades. The standard options for treatment of Laryngeal carcinoma are surgery, radiotherapy, chemotherapy or a combination of these. Radiotherapy is used in more than 70% of patients, surgery in about 55% and chemotherapy in 10%[149].

It is widely accepted that most early stage Laryngeal cancers can be adequately treated with single modality therapy, whether surgery or irradiation, with a 5 year local control of 85-98%.[150]. A multimodality approach based upon a combination of surgery and irradiation is the most common treatment for stage III and IV disease[132].

The development of oncologically sound conservation procedures has allowed more patients to retain airway and voice in early cancers. The refinement of radiation therapy techniques, potentially augmented by the addition of chemotherapy has allowed similar organ preservation with advanced cancers.

These improvements, however have not translated into significantly improved survival rates. Perhaps with an ever-increasing understanding of tumor biology and biologic response modifiers, immunotherapy may play a more prominent role in preventing the growth, spread and even the development of carcinomas.

3.2 Cell cycle

3.2.1 Basic concepts

The biology of cell division, differentiation and apoptosis is exceedingly similar in both normal and cancer cells. The cancer cell differs from its normal counterpart in that it is aberrantly regulated.

Cancer cells generally contain the full complement of bio molecules that are necessary for survival, proliferation, differentiation, cell death and expression of many cell type-specific functions. Failure to regulate these functions properly, however, results in an altered phenotype and cancer.

Four cellular functions tend to be inappropriately regulated in a neoplasm. First, the normal constraints on cellular proliferation are ineffective. Second, the differentiation program can be distorted. The tumor cells may be blocked at a particular stage of differentiation or they may differentiate into an inappropriate or abnormal cell type.

Third, chromosomal and genetic organization may be destabilized such that variant cells arise with high frequency; some variants may have increased motility or enzyme production that permits invasion and metastasis. Finally the tightly regulated cell death program (apoptosis) may be deregulated[151]

Cell division cycle can be divided into two functional phases- S and M phases, and two preparatory phases- G 1 and G 2. S phase is defined as the phase in which the DNA is replicated. The time it takes in a typical human cell to complete the S phase is about 8 hours and is invariant under normal circumstances.

Fully replicated chromosomes are segregated to each of the two daughter nuclei by the process of mitosis during M phase. The length of M phase is about 1 hour and is also normally invariant. G 1 phase precedes S phase, whereas G 2 phase precedes M phase.

G1 and G 2 phases are required for the synthesis of cellular constituents needed to support the following phase and ultimately to complete cell division. The length of G 2 phase in mammalian cells is about 2 hours. The length of the G 1 phase is highly variable and can range from about 6 hours to several days or longer.

In human cells, the varying length of G 1 phase accounts for most of the differences in time it takes to execute a cell division cycle between different cell types growing under different conditions. Cells that persist in G 1 phase for extended periods of time enter a distinct state called G 0.

Although such cells are metabolically active, they are not actively proliferating. Cells in G0 can re- enter the cell cycle or can remain in G0 indefinitely. A successful cell division cycle requires the orderly and unidirectional transition from one cycle phase to the next. Certain events must be completed before others are begun.

In theory, the ordering of cell cycle events may be accomplished in a manner analogous to the substrate product relationship of a metabolic pathway. The products of one reaction serve as the substrate for the next. The rate of first reaction, therefore, limits that of the next.

3.2.2 Cell cycle control

The timing and ordering of cell cycle transitions is dependent on separate positive and negative regulatory circuits. There are two classes of regulatory circuits; Intrinsic and extrinsic[152].

Intrinsic regulatory pathways are responsible for the precise ordering of cell cycle events. Because the length of S 1 G2 and M phases in mammalian cells is relatively invariant, intrinsic regulatory pathways control the transitions between these phases predominantly.

Extrinsic regulatory pathways function in response to environmental conditions. Both types of regulatory circuits can use the same checkpoints. Deregulation of intrinsic regulatory pathways can contribute to cancer.

For example, errors in the spindle assembly checkpoint can lead to chromosomal imbalance and anueploidy, a feature characteristic of virtually all cancers. Mis regulation of proteins that control this checkpoint has been detected in human cancer[153, 154]

Differences between normal and neoplastic cells are most commonly observed on the extrinsic regulatory circuits. Passage of cell cycle checkpoints ultimately requires the activation of intracellular enzymes known as Cyclin dependent kinases (CDKs).

CDKs exist in all eukaryotic cells from fungi to plants to mammals. Activation of CDKs is the central event in cell transitions; their activity is exquisitely regulated at several levels[155, 156]. Each Cyclin is synthesized at a particular stage of the cell cycle.

3.2.3 The G 0 to S checkpoint

The length of G 1 phase is highly variable and cells can exit the cell cycle for extended periods of time and mammalian cells do so during the G 1 phase of the cell cycle. Cells that have exited the cell cycle are said to be in G 0 state or quiescent. Most cells in adults are in G 0.

This absence from the cell division cycle can be temporary or permanent. These cells then re-enter the cell cycles, beginning a sequence of events that culminates in cell division. Hence the G o / G 1 to S phase regulation are highly regulated and the result of this regulation, by and large determines the growth fraction of a population of cells.

How re-entry into the cell cycle from G 0 is ultimately controlled?

Like other cell cycle transitions, activation of CDKs is required. G 0 cells are devoid of significant CDK activity. In the presence of mitogenic growth factors, expression of D type Cyclins (Cyclin D 1 D 2 D 3) is stimulated and continues throughout G 1 phase as long as growth factors are present[157].

D type Cyclins complex with either CDK 4 or CDK 6 catalytic subunits to form a holoenzyme modified by CDK activating kinase (CDK).One important substrate is the retinoblastoma tumor suppressor protein (Rb) and other members of its gene family p107 and p 130.

Rb is constitutively expressed and constrains cells from progressing through the G 1 phase of the cell cycle[158]**.**On phosporylation by Cyclin D / CDK 4 or 6, Rb loses its ability to restrain the cell cycle[159]. Expression of wild type Rb complementary DNA (cDNA) in cancer cells can inhibit their tumorigenicity[160].

Cyclin D / CDK 4 or 6 phosphorylation, which, in turn is regulated by p16 INK 4a, inhibits Rb function. Tumor cells that lose p 16 INK 4a or over express Cyclin D 1 generally retain wild type Rb. Cells lacking wild type Rb typically express Cyclin D 1 and p16 INK 4 a. in addition, induction of cell cycle arrest by forced expression of p 16 INK 4 a occurs only in cells that contain function Rb.

If mutations in any of the members of this pathway are considered, disruption of this p16 INK 4a/ Cyclin D1/ CDK4 or 6/Rb pathway may occur in most human cancer[161]. Because this pathway is important for regulation of the G0 to S phase transition, it has a major influence of the growth fraction of normal tissues.

Like normal cells, the transit of cell cycle check points in cancer cells ultimately requires the activation of CDKs. Owing to the complexity of CDK regulation, defects leading to inappropriate activation of CDks can occur at several levels.

The over expression of Cyclin D 1 has been detected in many human cancers owing to gene amplification or translocation of the Cyclin D1 gene[101]. The Cyclin D1 gene is located on chromosome 11 q 13. Cyclin D 1 gene aberrations are frequently detectable in head and neck tumors, especially in carcinomas of the hypo pharynx and, to a lesser extent, in those of the oro pharynx and larynx.

Alterations in Cyclin D 1 (in the earliest phases, over expression, and later CCND 1 amplification) and high telomerase activity are early events in laryngeal cancer; these may be potential markers for the prediction of neoplastic progression.

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Cyclin D1 is a growth factor responsive Cyclin that plays an important role in regulating G0 /S checkpoint deregulated expression of Cyclin D1 could inappropriately increase Cyclin D 1/CDk 4 activities and drive transit of the checkpoint even in the absence of appropriate growth factors.

Direct evidence that forced expression of Cyclin D 1 can facilitate tumorigenisis has been obtained from transgenic mice in which over expression of Cyclin D1 has been targeted to the mammary epithelium. These mice develop ductal hyper proliferation and eventually mammary tumor formation.

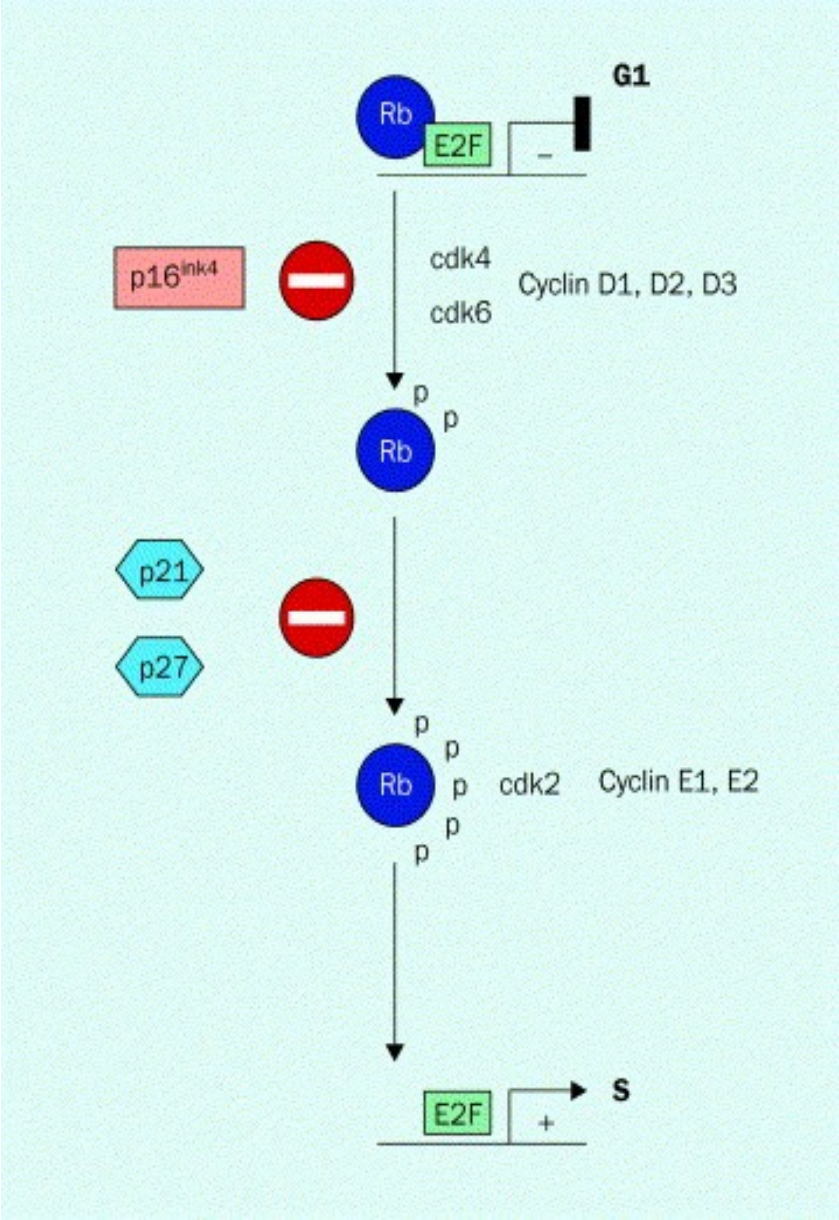

Fig. 2 Factors regulating the restriction point in the G1 to S phase of the cell cycle

cdk inhibitors such as the p16ink4 family and the p21 family restrict the activity of cyclin-D-dependent kinases and cyclin-E dependent kinases respectively. Progressive Rb phosphorylation leads to liberation of active E2F to induce transcription of S-phase genes

3.3 Cyclins

Cyclins are a class of structurally related proteins that bind and activate the catalytic subunit of cyclin dependent kinases (CDKs). There are eight type of cyclins, (cyclin A to H) and all of them share an ~ 150 amino acid region of homology called the cyclin box whish is responsible for the CDK binding and activation[162].

Cyclins can be roughly divided into two subfamilies: the G 1 Cyclins and the mitototic Cyclins. The G1 Cyclins (C, D, E) are short lived and have rapid turnover throughout cell cycle, levels of transcription of their m RNA determine their levels.

The mitotic Cyclins (A, B) are very stable throughout the interphase but undergo rapid proteolysis by an ubiquitin dependent pathway during mitosis. Compared to the mitotic Cyclins, the G1 Cyclins have a longer C terminal sequence after Cyclin box, and it is this part of the protein that seem to confer instability to the G1 Cyclins.[163]

The function of Cyclins is primarily controlled by changes in the Cyclin levels, which increase at specific stages and are categorized by the stage at which they are expressed. The G 1 Cyclins differ from the mitotic Cyclins in their overall primary structure and this has implications for their stability during the cell cycle.

The transcription of the mitotic Cyclins, Cyclin A and B are cell cycle dependent and their levels are determined by transcription and proteolysis[164]. Protein deregulation is an effective method for promoting unidirectional cell cycle transitions because of its rapidity and irreversibility.

Three major cell cycle transitions, entry into S phase, separation of sister chromatids and exit from mitosis, require the degradation of specific proteins via the ubiquitination by 26 s proteosome pathways. The degradation of mitotic Cyclins involves the ubiquitin dependent proteolytic machinery and a small sequence motif called the destruction box located at the N terminus.

This region has a small cluster of conserved residues followed by a lysine rich stretch. The destruction box region differs between Cyclin A and B and probably accounts for the finding that in mitosis Cyclin A is degraded before Cyclin B.

G1 Cyclins lack the mitotic destruction box but contain the PEST sequences as the peptide motifs that are important for proteolysis. PEST sequences (rich in proline, glutamic acid, serine and threonine) are frequently present in unstable proteins such as G 1 Cyclins and contain specific sites of phosphorylation.

Phosphorylation of PEST regions facilitates their destruction of PEST sequence proteins. Specific motifs within them actually control the PEST sequence by the ubiquitination machinery.[165]

Cyclins are thought to target the CDK to specific substrate and sub cellular locations. Cyclin expression varies during cell cycle and the periodic expression of different Cyclins defines the start of each phase of the cell cycle and also marks the transition between the various phases.

Cyclins and these cognate CDK catalytic subunits non-covalently form 1:1 complexes to produce the CDK holoenzyme. Specific CDKs operate in distinct phases of cell cycle.

Cyclin dependent kinases

The major transitions of the eukaryotic cell cycle are triggered be a family of serine kinases called the Cyclin dependent kinases (CDKs). At least nine CDKS (CDK 1-9) are known so far[166].

CDK activation requires the regulatory subunit called the cyclins and phosphorylation of a conserved throenine by the CDK-activating kinase(CAK) which itself is a complex of a regulatory Cyclin H and a catalytic CDK 7 subunit.

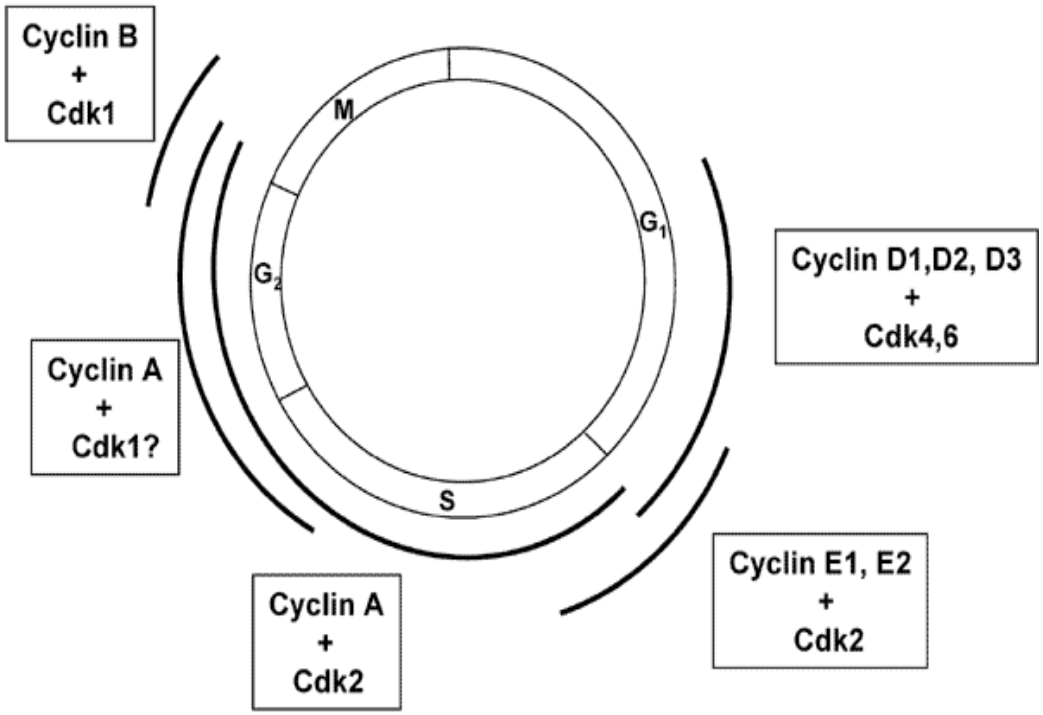

Fig. 3 Cyclin-dependent kinase (cdk) function in the cell cycle

D-type Cyclins (Cyclins D1, D2, and D3) activate cdk4 and cdk6 for functions extending from mid G_1 to the G_1/S-phase transition. E-type Cyclins (Cyclins E1 and E2) activate cdk2 for functions at the G_1/S-phase boundary, probably extending into early S phase. Cyclin A activates cdk2 for functions extending from the G_1/S-phase boundary and extending into G_2. Cyclin A is known to interact with cdk1 as well; however, no specific function for this complex has been identified. Finally, Cyclin B activates cdk1 at the G_2/M-phase boundary with activity that lasts until Cyclin B is degraded during anaphase.

Cellular CDK levels tend to remain in constant excess throughout the normal cell cycle and catalytic activity of CDKs is regulated post translationally. CDKs are closely related in size and sequence (>40% identity).

In human cells, the growing list of CDKs includes the confounding member, CDC 2 and CDK 2- CDK 7. Our understanding of CDK structure and function is based largely on studies of prototypical CDKs of S. Pombe (cdc 2)

The typical CDK catalytic unit contains 300 aminoacids. The catalytic subunit is completely inactive when it is monomeric and unphosphorylated. Cyclins are the primary regulators of the CDKs.

3.3.1 Cyclin dependent kinase inhibitors

The Cyclin-CDK complexes are in turn regulated with small inhibitory,endogenous family of proteins termed Cyclin dependent kinase inhibitors (CKIs) that bind and inactivate CDK Cyclin complexes. Two families of inhibitors have been identifies in mammalian cells, with different modes of action.

One group comprising related proteins namely p 21 cip1, p 27 kip 1 and p 57 kip 2. They appear to function as broad specificity inhibitors of Cyclin/CDK complexes. They associate with a complex containing a Cyclin, a CDK and proliferating cell nuclear antigen (PCNA).

The second family of inhibitors has four members in the family p 15, p16, p18and p19. These CKIs act as competitive inhibitors of D type Cyclins by forming specific complexes with the D type Cyclin partners, CDK 4 and CDK 6.[167]

Although members of the kinase inhibitor proteins were initially thought to exclusively regulate G1 and S phases, several reports have demonstrated that these proteins also regulate G2/M phase transition.

Table -1

Cyclins and cell cycle phases

Cyclin	Cell cycle phase
Cyclin D1	**Early G1 phase**
Cyclin E	**G1/S transition**
Cyclin A	**S phase**
Cyclin B	**G2/M phase**

Cyclin D

The first group of Cyclins that is expressed after the cells are stimulated to enter the cell cycle is the D type Cyclins. D type Cyclins act as growth factor sensors. Cyclin D has a relatively short half-life of 20 minutes approximately and rapidly disappears with the removal of mitogenic stimuli or the addition of antiprolifetative agents.

Cyclin D complexed with respective partners CDK 4 and CDK 6, participates in the transduction of external signals (mitogenic or antiproliferative) to other components of G1/S transition cell cycle machinery. Cyclin D helps in moving G0 cells into G 1 and early G 1 cells into the G 1/ S transition in response to the extra cellular stimuli.

The D type Cyclins belong to a distinct subset within Cyclin family based on structural and functional criteria. There are three genes identifies under this family, Cyclin D 1, D2 and D 3[168].

Table -2

Cyclin D gene and chromosome locus

Gene	Chromosome Locus
CCND 1 (cyclin D 1)	11 q 13
CCND 2 (cyclin D 2)	12 p 13
CCND 3 (cyclin D 3)	6p 21

The D type Cyclins contain the sequence Leu-X-Cys –X-Glu near their amino terminus. This sequence is common to DNA viral oncoproteins SV 40 T antigen, adenovirus E 1 and human papilloma virus E 7 that binds and inactivates p RB and p RB related proteins.

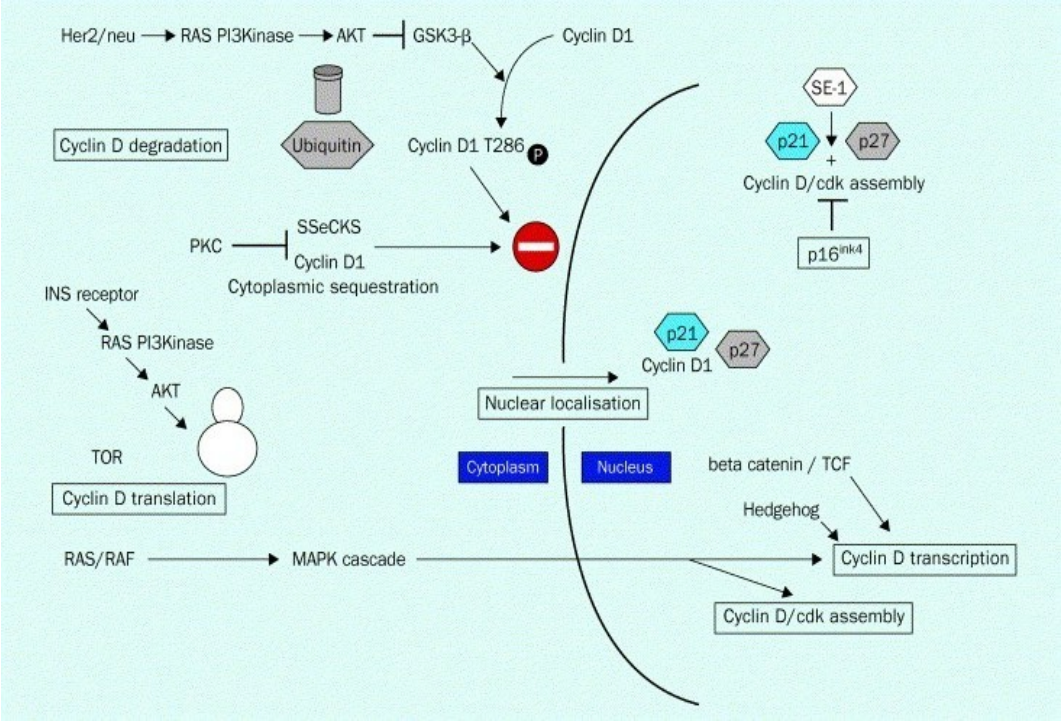

Fig. 4 Cyclin D regulation

Ubiquitination of cyclin D protein is regulated by GSK3-β-mediated phosphorylation. Cyclin D/cdk complexes require p21, p27, and SE-1 to promote their assembly. Ras pathways intervene at several points to promote translation, transcription, assembly of complexes and the inhibition of cyclin D degradation.

The D type Cyclins bind directly to p Rb and p 107 invitro and the interactions are disrupted by point mutations of the Leu-X-Cys –X-Glu motif. The oncogene-derived peptides that contain this motif compete with Cyclin D to bind to pRb[169].

Cyclin D 2 and D 3 form more stable complexes than Cyclin D 1 suggestive of differences in functional interaction of Cyclin D with pRb underscores its role as a positive growth regulator with oncogenic potential.

3.3.2 p16/ pRb/Cyclin D 1 pathway

The emerging critical role of the p16/pRb/Cyclin D 1 pathway in cell cycle regulation is supported by frequent aberrations of the individual components of this checkpoint mechanism in human tumors. Cyclin D 1 has the cell cycle accelerating property, which is mediated by neutralizing the froth restraining property of p Rb via its phosphorylation.

This function of Cyclin D1-CDK 4 complex is antagonized by p 16, the tumor suppressor. This pathway has an undisputable biological significance because the whole pathway as one functional unit I s a single complex target of oncogenic alterations.

Abundant evidence as been accumulated that suggests identification of a precise aberrant component within this pathway can have prognostic, diagnostic and potentially therapeutic implications.

G1 accelerating function of Cyclin D 1 and the growth restraining function of p16 however require a functional retinoblastoma protein. Alterations in this pathway have been reported in non-small cell lung cancers[170] and also in sporadic malignant melanoma.

3.4 Studies on Cyclin D1 as prognostic marker in Head and neck squamous cell carcinomas

p53 and Cyclin D1 as prognostic factors in squamous cell carcinoma of the larynx.[171]

Proteins p53 and Cyclin D1 play a crucial role in cell cycle control. Protein p53 mutations are one of the most common genetic alterations in human cancer, and cyclin D1 gene amplification has been found to be associated with poor prognosis in different types of tumors.

Functional alterations of these proteins may play an important role both in the carcinogenesis of squamous carcinomas of the head and neck and in the clinical evolution of these tumors. Presence of immunohistochemically detectable p53 is associated with shorter survival and disease-free intervals.

Multivariate statistical analysis revealed that the accumulation of p53 is an independent prognostic factor, which is associated with shorter survival. This association was not evident in the case of Cyclin D1.

Overexpression of Cyclin D1 indicates a poor prognosis in squamous cell carcinoma of the head And neck[172]**.**

Overexpression of cyclin D1 was not associated with known prognostic factors (eg, the T and N stages). Tumors recurred more frequently and in a shorter period in patients whose primary tumors showed an over expression of cyclin D1 protein. This difference (P = .05) was statistically

significant in a stepwise proportional hazard regression analysis. .Whether over expression of cyclin D1 may therefore be used to select patients for more intensive treatment should be examined in the context of a clinical trial.

Prognostic significance of p53, bax and bcl-2 gene expression in patients with laryngeal carcinoma[173].

The expression of bcl-2 gene appears to be an independent prognostic factor for patients with laryngeal carcinoma. The co expression of the genes studied can be used to determine aggressive clinical phenotypes.

Prognostic significance of expression of p53, bcl-2 and bax in squamous epithelial carcinoma of the larynx--a multivariate analysis[174]

Conventional clinico pathologic parameters do not accurately reflect the clinical outcome of patients with head and neck carcinoma. The establishment of additional prognostic factors that may give insight into the biologic features of a tumor is therefore an essential goal.The present study analyses the expression patterns of p53, bcl-2, and bax with regard to their relationships with conventional tumor parameters and to their prognostic significance in patients with laryngeal squamous cell carcinoma.

Bcl-2 immunoreactivity was positively correlated with an advanced clinical stage, a high T category, regional lymph node metastasis, and a high histological grading. Significant relationship between clinicopathologic parameters and p53 or bax expression were not detectable. The age of the

patients, advanced disease, positive bcl-2 expression, and a high level of p53 expression were significantly associated with shortened disease-specific survival in univariate analysis.

In multivariate analysis, age, clinical stage, and p53 expression had independent prognostic value. Although expression of p53 and bcl-2 was found to be clinically relevant in univariate analysis, only p53 but not bcl-2 was an independent predictor of patient outcome.

This superiority of p53 in multivariate analysis points to its central role within cell cycle and death regulation, with which it influences two important parameters of tumor progression.

p53, Ki67 and Cyclin D1 as prognosticators of lymph node metastases in laryngeal carcinoma[175].

The prognosis in patients suffering from head and neck squamous cell carcinomas depends on many factors. However, regional lymph node metastases are the most important parameter in determining the cure and survival of patients with head and neck cancers.

The evaluation of cancer cell biology enables differentiation of their proliferation and tendency of metastases. Immunohistochemical examinations complement the well-established routine histological examination. The aim of this study was to evaluate the prognostic importance of the level of immunoproliferating proteins such as Cyclin D1, nuclear antigen Ki67 and suppressor gene p53 for regional lymph node metastases in laryngeal carcinoma.

No statistically significant correlation was found between staining intensity of suppressor gene p53, Cyclin D1 and the degree of local advancement (T). There was no correlation between the level of immunoproliferative markers and regional lymph node metastases.

Statistically significant correlation was found between T stage and staining for Ki67 as well as between Cyclin D1 level and Ki67.In conclusion, (1) no significant correlation was found between Ki67 and Cyclin D1, p53 and TNM classification; (2) lack of correlation was confirmed between N+, p53, Ki67, Cyclin D1; (3) the degree of histological grading correlated with Cyclin D1 expression.

Cyclin D1 expression is predictive of occult metastases in head and neck cancer patients with clinically negative cervical lymph nodes[176].

The aim of this study was to investigate the value of p53 and Cyclin D1 gene expression in predicting the risk of occult lymph node metastases in patients with head and neck squamous cell carcinoma. The expression of Cyclin D1 and p53 was evaluated by means of immunohistochemical analysis.

This study indicates that the expression of Cyclin D1 correlates with the presence of occult cervical metastases in head and neck carcinoma patients, thus suggesting that its immunohistochemical evaluation in biopsy samples may be used as an additional tool for identifying patients to be treated with elective neck dissection .

Image cytometry of Cyclin D1: a prognostic marker for head and neck squamous cell carcinomas[177].

CCND1 gene amplification and Cyclin D1 protein over expression are indicators for poor prognosis in invasive head and neck carcinomas. Increased CCND1 gene dosage is a more sensitive prognostic factor than protein over expression as evaluated by conventional immunohistochemical techniques.

Qualitative immunohistochemistry cannot distinguish Cyclin D1 over expression accompanied by amplification of the CCND1 gene from over expression associated with normal CCND1 gene copy number. To improve the sensitivity of Cyclin D1 protein determination, quantitative techniques of image analysis were applied to evaluate Cyclin D1 gene amplification in head and neck carcinomas.

There was a significantly higher rate of occurrence of adverse events among patients with CCND1 gene amplification than among those without gene amplification. There was a strong association between CCND1 gene amplification (as detected by Southern blot analysis) and the highest nuclear score (by image cytometry of the immunostained tumor sections).

The predominance of cells in the lowest nuclear score category was significantly associated with normal copy number .Conversely, the highest nuclear score was a significant predictor of gene dosage .Similarly, high nuclear score was a good predictor of death as the final outcome of the disease.

Although somewhat less accurate than Southern blotting, image cytometry of immuno histochemical Cyclin D1 stain appears to be a promising tool that could be useful for other tumor marker expression studies.

Cyclin D1 amplification and p16 (MTS1/CDK4I) deletion correlates with poor prognosis in head and neck tumors[178].

Cyclin D1, a cell cycle regulator localized to chromosome 11q13, is amplified in several human tumors including head and neck squamous cell carcinoma (HNSCC). Amplification and/or over expression of Cyclin D1 have been correlated to a poor prognosis. Deletion of the p16 gene, localized to 9p21, has also been observed in a significant proportion of HNSCC. The p16 gene regulates Cyclin D1-CDK4 activity and prevents retinoblastoma tumor suppressor gene phosphorylation, thereby down regulating cellular proliferation. FISH is a simple and sensitive method for detecting Cyclin D1 amplification and p16 deletion in head and neck cancer. Our results suggest that these two genetic aberrations together portend a poorer outcome than either of the abnormalities alone in head and neck cancer.

Inactivation of p53 and amplification of cyclin D1 in squamous cell carcinomas of the head and neck[179].

P53 and CCND1 (Cyclin D1) genes play a critical role in the cell cycle regulation. Abnormalities of these genes are frequent in different types of cancers, including those of the head and neck. The aim of this work is to investigate whether P53 inactivation (determined by loss of heterozygosity

analysis) is related to CCND1 gene amplification (determined by differential PCR analysis). No relationship was found between P53 inactivation, the clinico-pathological parameters analyzed and the clinical outcome. CCND1 amplification was associated with advanced T-stages, nodal metastases and a decreased survival. The combination of both abnormalities shows a pattern that seems to be additive, since it was associated with an increase in tumor recurrences and a decrease in survival that was higher than for either of them individually .

Cyclin D1 gene amplification in human laryngeal squamous cell carcinomas: prognostic significance and clinical implications[88].

The Cyclin D1 (CCND1) gene is amplified, rearranged, and overexpressed frequently in human cancer, including squamous cell carcinoma. CCND1 amplification did not correlate with age, tumor localization and extension, cervical lymph node involvement, histopathological grading, and epidermal growth factor receptor levels.

In a univariate analysis, CCND1 amplification, tumor extension, lymph node involvement, poor histological differentiation, and high epidermal growth factor receptor levels were correlated significantly with shorter overall survival. In a multivariate analysis, only CCND1 and tumor extension retained statistically significant prognostic values.

This is the first report in which CCND1 amplification is identified as a significant independent prognostic factor in laryngeal carcinoma. Evaluation of CCND1 amplification could be applicable to the clinical management of laryngeal cancer, allowing identification of patients with poor prognoses .

Expression patterns of Cyclins D1, E in laryngeal epithelial lesions: correlation with other cell cycle regulators (p53, pRb, Ki-67 and PCNA) and clinicopathological features[180].

The expression of cell-cycle progression molecules Cyclin D1 and Cyclin E were immuno histochemically examined. The results of their expression were compared with two cell-cycle implicated tumor suppressor proteins p53 and pRb as well as with two proliferation associated indices PCNA and Ki-67 in an attempt to elucidate their potential role in the pathogenesis and progression of laryngeal epithelial lesions.

High levels of Cyclin D1 and E expression were correlated with increased Ki-67 score. A significant positive correlation between Cyclin D1 and E was also detected in carcinomas. Decreased levels of Cyclins D1 and E in the group of in situ carcinomas compared with those of dysplastic cases and papillomas were also observed. In the dysplastic lesions Cyclin D1 expression was correlated with pRb expression.

In the cases of keratosis cyclins D1 and E expression were correlated with pRb, while Cyclin D1 was associated with PCNA and Ki-67 score. In conclusion the expression of Cyclins D1 and E in squamous cell carcinomas of the larynx does not seem to have a prognostic significance. In addition, their expression may be involved in the development of laryngeal lesions, implicated in cell proliferation, with other cell cycle related proteins, probably by different molecular pathways.

Overexpression of Cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck[181].

Overexpression of Cyclin D1 was also associated with a shortened overall survival of these patients with a 5-year survival of 60% for the Cyclin D1 strongly positive cases and of 83% for Cyclin D1-negative cases. Overexpression of Cyclin D1 appears to indicate poor prognosis

Molecular and immuno histochemical analysis of the bcl-1/cyclin D1 gene in laryngeal squamous cell carcinomas: correlation of protein expression with lymph node metastases and advanced clinical stage[182].

The molecular pathogenesis of laryngeal squamous cell carcinomas (LSCCs) is still only partially understood, although genetic alterations affecting various proto oncogenes or tumor suppressor genes have often been detected. To

improve their understanding of the role of Cyclin D1 in the pathogenesis of LSCCs, the authors investigated the expression of Cyclin D1 protein and the amplification status of the bcl-1/cyclin D1 locus in a panel of 58 pathologic samples. The authors found almost complete concordance between locus amplification and protein expression. Statistical analysis showed a correlation between Cyclin D1 expression and both the presence of lymph node metastases and advanced clinical stage. The authors' observations suggest that the deregulation of Cyclin D1 expression may be involved in the pathogenesis of more aggressive LSCCs .

PRAD-1/cyclin D1 gene amplification correlates with messenger RNA over expression and tumor progression in human laryngeal carcinomas[183].

PRAD-1 is a putative oncogene localized on chromosome 11q13 which encodes Cyclin D1, a novel Cyclin involved in cell cycle regulation. Amplification of this gene has recently been reported in several human tumors including breast and head and neck carcinomas.

In this study we have analyzed the presence of PRAD-1/cyclin D1 gene amplification and mRNA over expression in a series of 46 matched normal mucosas and squamous cell carcinomas of the larynx. DNA amplification correlated with advanced local invasion), presence of lymph node metastases, and stage IV of the tumors.

mRNA over expression was also significantly associated with advanced local invasion and stage IV carcinomas. A significant association was observed

between gene amplification and mRNA over expression .Furthermore, the degree of DNA amplification correlated with the levels of mRNA expression.

These findings suggest that the PRAD-1/cyclin D1 gene may be an important target of 11q13 amplifications in laryngeal carcinomas and the activation of this gene may be involved in the progression of these tumors. Its association with advanced-stage tumors indicates that PRAD-1/cyclin D1 gene amplification and over expression may be of prognostic significance.

Clinical relevance of Cyclin D1 protein over expression in laryngeal squamous cell carcinoma[87].

Cyclin D1 over expression was significantly associated with tobacco smoking and alcohol consumption, tumor extension, advanced clinical stage, and the presence of lymph node metastases.

Univariate analysis showed that a shorter disease-free and overall survival were significantly associated with supraglottic site, tumor extension, advanced clinical stage, and Cyclin D1 over expression. At multivariate analysis, tumor extension and Cyclin D1 over expression were significantly associated with tumor recurrence, whereas tumor extension, supra glottic site and, at a borderline level of statistical significance, Cyclin D1 over expression, were associated with reduced overall survival.

The over expression of Cyclin D1 in LSCC is associated with unfavorable clinico pathologic features and represents an independent significant predictor of laryngeal carcinoma prognosis, particularly for disease-free survival. This indicates that Cyclin D1 evaluation may be a further useful element for selecting subgroups of patients who should be treated with more aggressive therapies.

Cyclin-D1-gene amplification is a more potent prognostic factor than its protein over-expression in human head-and-neck squamous-cell carcinoma[48].

To evaluate the prognostic significance of Cyclin D1 protein/gene expressions in human head-and-neck squamous-cell carcinoma, we examined amplification of the cyclin-D1 gene (CCND1) by the differential PCR method and over-expression of cyclin-D1 protein by immuno histochemistry.

The overall 5-year survival of patients with CCND1 amplification or with protein over-production was significantly lower than that of patients without. However, with multivariate analysis, only amplification of CCND1 retained an independent prognostic value .These suggest that CCND1 amplification occurs at early stages of HNSCC tumorigenesis and is a more useful prognostic factor than over-expression of CyclinD1 in HNSCC

Cyclin D 1 : Target for therapeutic strategies

Antisense Cyclin D1 enhances sensitivity of head and neck cancer cells to cisplatin[184].

Cyclin D1 is a cell cycle regulatory factor that modulates a critical step in cell cycle control. Cyclin D1 is over expressed in a significant proportion of head and neck cancers and correlates with a poor prognosis. Abrogation of Cyclin D1 action through antisense Cyclin D1 shows promise as an anti tumor therapy, with an inhibitory effect in head and neck squamous cell carcinoma both in vitro and in vivo.

The suppressive effect of antisense Cyclin D1 in head and neck cancer xenografts in nude mice is incomplete, however, suggesting that combination with another antitumor agent is necessary for complete tumor eradication. Cisplatin is a widely used chemotherapeutic agent in head and neck cancer, and is particularly effective in combination with radiation therapy.

In this study, we investigate whether antisense Cyclin D1 enhances the sensitivity of head and neck cancer cells to cisplatin. Such an enhancement of sensitivity would suggest that combination therapy using antisense Cyclin D1 and cisplatin would be an effective treatment modality for head and neck cancer.

Increasing concentrations of cisplatin resulted in significantly higher rates of cell killing in the antisense Cyclin D1-transfected cells than in the parental cells.. Western blot analyses demonstrated decreased expression of Cyclin D1 in

the CCL23AS cells with increasing doses of cisplatin, compared with the parental CCL23 cells.

Antisense Cyclin D1-transfected CCL23 cells demonstrate an enhanced sensitivity to the effects of cisplatin compared with the parental cell line. Although the mechanism for this phenomenon is not completely understood, the data suggests the potential use of combination therapy using antisense Cyclin D1 and cisplatin for head and neck cancers.

While neither agent alone can completely eradicate head and neck cancers, the synergistic effect of the two may be an effective therapeutic protocol for refractory head and neck cancers. Future investigation into the combination of antisense Cyclin D1 with cisplatin for treatment of head and neck cancer is needed.

Prognostic significance of P27 and Cyclin D1 co-expression in laryngeal squamous cell carcinoma: possible target for novel therapeutic strategies[165].

Tumor cells are characterized by uncontrolled growth due to alterations in the genes that play a key role in cell repair systems and apoptosis: pro-mitotic oncogenes such as Cyclin D1, and tumor suppressor genes such as p27. Recent studies have demonstrated that these genes are involved in different epithelial neoplasms and that their expression is generally associated with prognosis. Multivariate analysis showed that Cyclin D1 and p27 were the only statistically significant predictors of disease-free and overall survival.

In relation to the simultaneous expression of p27 protein and Cyclin D1, the patients with a Cyclin D1+/p27-phenotype had the poorest disease-free and overall survival rates. On the basis of these immuno histochemical results, it was possible to select a subgroup of patients with a high risk of recurrence and poor prognosis to undergo more extended surgical treatment and/or combination anti tumoral therapeutic procedures.

CHAPTER 4

RESULTS

This study was done by Immuno histochemically. The controls used were sections from squamous cell carcinoma of larynx known to over express Cyclin D1. The negative control had omission of the primary antibody. Only nuclear staining of cells was taken as positive. Whole of the stained section was studied microscopically under 40 X magnification.

Scores were ranked as – negative; +/- 0-5 % tumor cells were positive; + 5-50% of tumor cells were positive; ++ >50 % of tumor cells were positive.[87,107,181]

4.1 Cyclin D 1 expression in normal laryngeal epithelium

Normal laryngeal epithelium does not express Cyclin D 1 or sometimes weak staining may be present.

i. Cyclin D 1 expression in laryngeal cancer

In head and neck squamous cell carcinoma the most commonly studied Cyclin has been Cyclin D 1. 35-64% of head and neck cancers[87, 111,172,181] have been reported to over express Cyclin D 1 or to have CCND 1 gene amplification.

Table 3

CLINICOPATHOLOGICAL PARAMETERS

Variable	Number N=74	Percentage
Sex		
Male	71	95.9%
Female	3	4.1%
Age		
< 60 years	22	29.8%
> 60 years	52	70.2%
Subsite		
Supra glottis	15	20.3%
Glottis	47	63.5%
Trans glottis	12	16.2%
T Status		
T 1a	12	16.2%
T 1b	6	8.1%
T 2	24	32.4%
T 3	17	23.0%
T 4	15	20.3%
N status		
N 0	50	67.6%
N 1	7	9.5%
N 2a	1	1.4%
N 2b	9	12.2%
N 2c	5	6.8%
N 3	2	2.7%

Table 4

Variable	Number N=74	Percentage
Stage		
1	16	21.6%
2	15	20.3%
3	13	17.6%
4	30	40.5%
Grade		
G 1	7	9.5%
G 2	25	33.8%
G 3	35	47.3%
G x	7	9.5%
G x Grade not known		
Treatment		
Radiotherapy only	51	68.9%
Surgery only	4	5.4%
Surgery and post operative Radiotherapy	17	23.0%
Radiotherapy and salvage Surgery	2	2.7%

Relationship between age and cyclin D 1 expression

AGE(years)	CyclinD1expression			
	Negative	Percentage	Positive	Percentage
<30	1	2.0	0	0.0
31- 40	3	5.9	1	4.3
41-50	11	21.6	6	26.1
51-60	18	35.3	6	26.1
61-70	12	23.5	7	30.4
71-80	6	11.8	3	13.0

P value = 0.9253

Table 5

Relationship between subsite and cyclin D 1 expression

SUBSITE	Cyclin D1 expression			
	Negative	Percentage	Positive	Percentage
Supra glottis	9	17.6	6	26.1
Glottis	33	64.7	14	60.9
Trans glottis	9	17.6	3	13.0

P value = 0.6700

Table 6

Relationship between T status and cyclin D 1 expression

T STATUS	Cyclin D1 expression			
	Negative	Percentage	Positive	Percentage
T 1a	6	11.8	6	26.1
T 1b	3	5.9	3	13.0
T 2	16	31.4	8	34.8
T 3	11	21.6	6	26.1
T 4	15	29.4	0	0.0

P value = 0.0409, significant

Table 7

Relationship between N status and cyclin D 1 expression

N STATUS	Cyclin D1 expression			
	Negative	Percentage	Positive	Percentage
Node negative	36	70.6	14	60.9
Node positive	15	29.4	9	39.1

P value = 0.5766

Table 8

Relationship between stage and cyclin D 1 expression

STAGE	Cyclin D1 expression			
	Negative	Percentage	Positive	Percentage
I & II	23	45.1	7	30.4
III & IV	28	54.9	16	69.6

P value = 0.3506

Table 9

Relationship between grade of tumor and cyclin D 1 expression

GRADE	Cyclin D1 expression			
	Negative	Percentage	Positive	Percentage
G 1	5	9.8	2	8.7
G 2	19	37.3	6	26.1
G 3	23	45.1	12	52.2
G x	4	7.8	3	13.0

P value = 0.7465

Table 10

Distribution of mean and standard deviation of time (in months) to recurrence and cyclin D 1 expression

Cyclin D 1 expression	Number of recurrences	Mean ± SD	Median	P value
Negative	6	8.8 ± 6.0	8.0	0.247
Positive	4	18.3±17.5	12.5	

Table 11

Distribution of mean and standard deviation of time (in months) to residual disease after primary radiotherapy and cyclin D 1 expression

Cyclin D 1 expression	Number of residues	Mean ± SD	Median	P value
Negative	5	6.8 ± 2.6	8.0	0.699
Positive	8	7.6 ± 4.1	7.5	

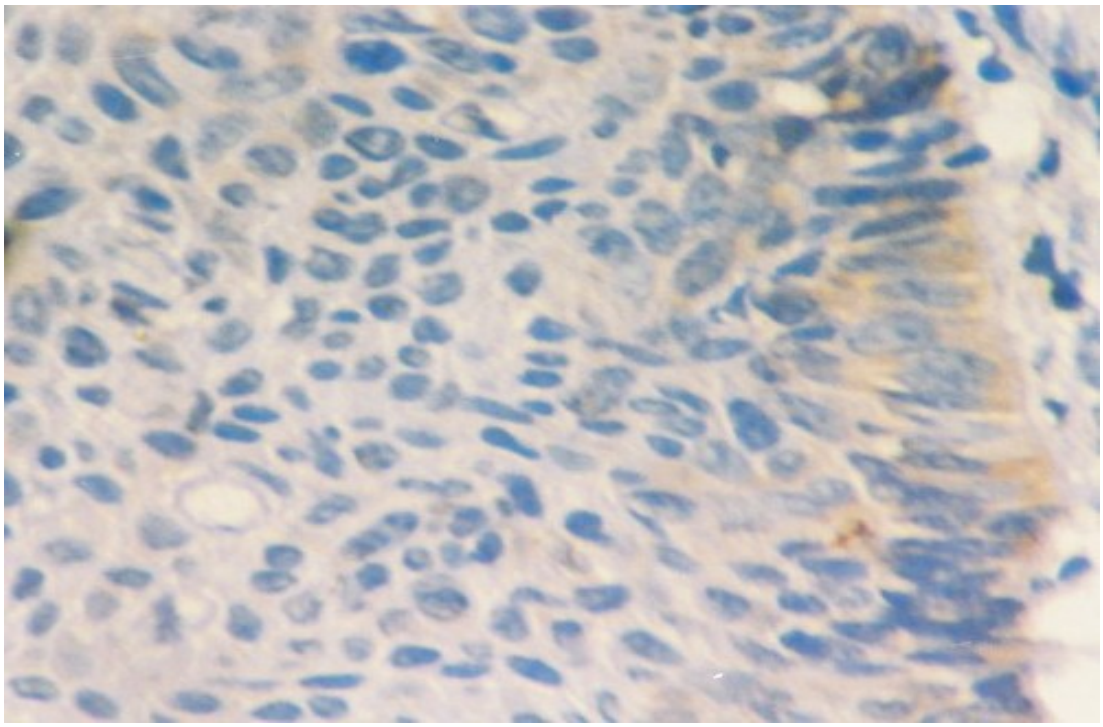

Fig. 5 Squamous cell carcinoma of larynx- Cyclin D1 expression is negative

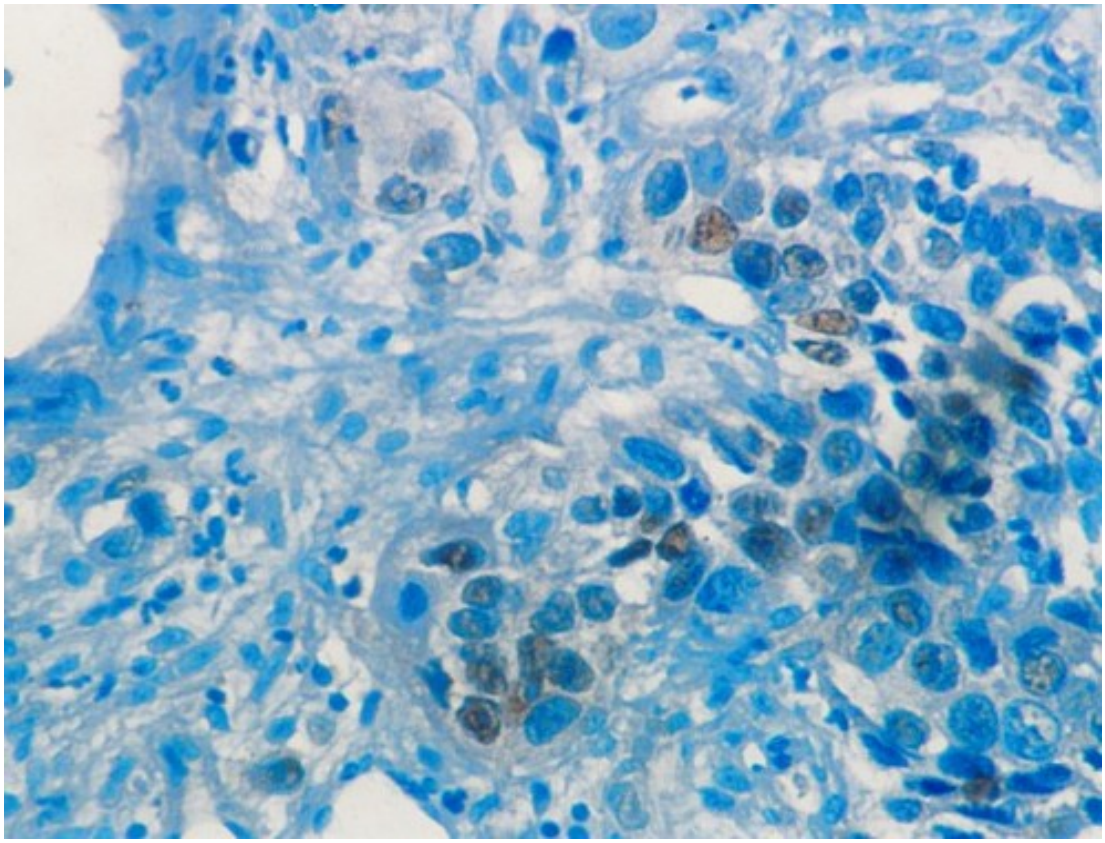

Fig. 6 Squamous cell carcinoma of larynx - Cyclin D1 expression is 10 percent

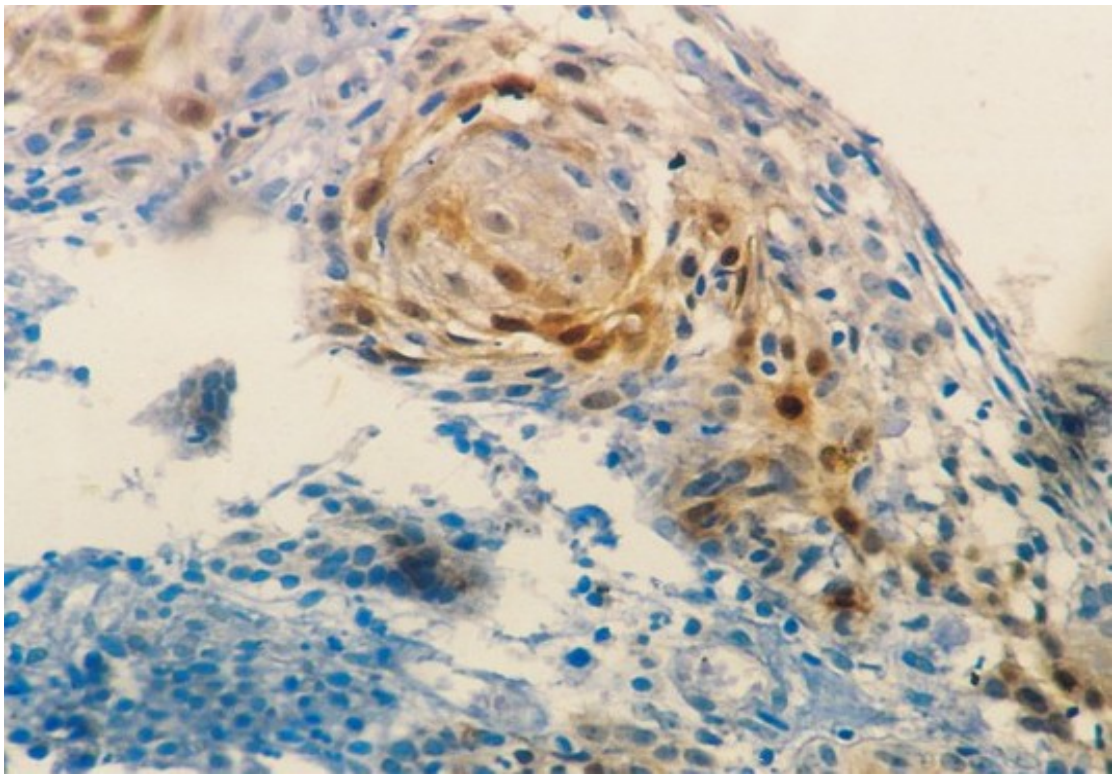

Fig.7 Squamous cell carcinoma of larynx - Cyclin D1 expression is 20 percent

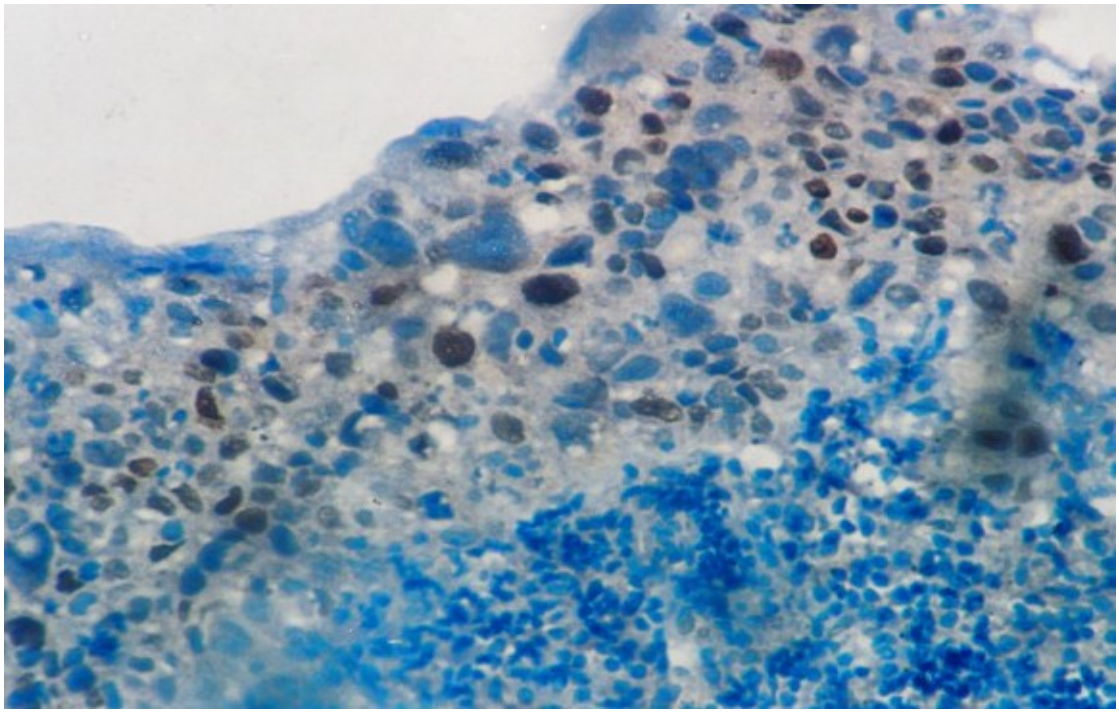

Fig. 8 Squamous cell carcinoma larynx-Cyclin D1 expression is 30 percent

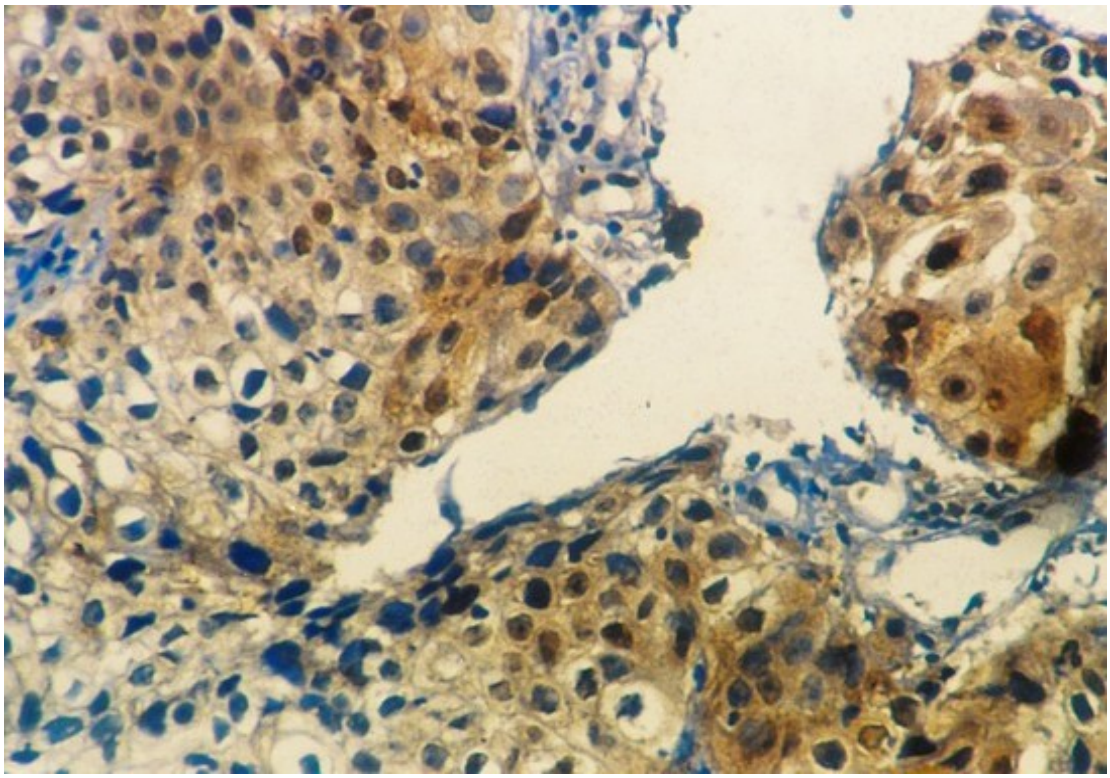

Fig.9 Squamous cell carcinoma of larynx- Cyclin D1 expression, 50 percent

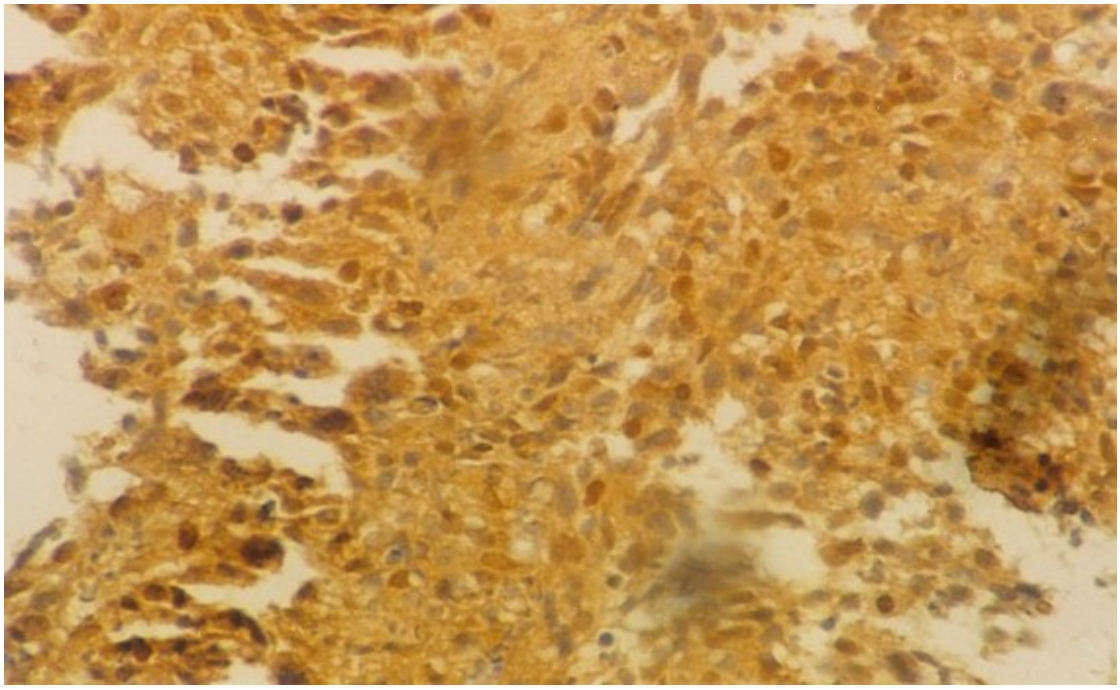

Fig.10 Squamous cell carcinoma of larynx-Cyclin D1 expression, 80 percent

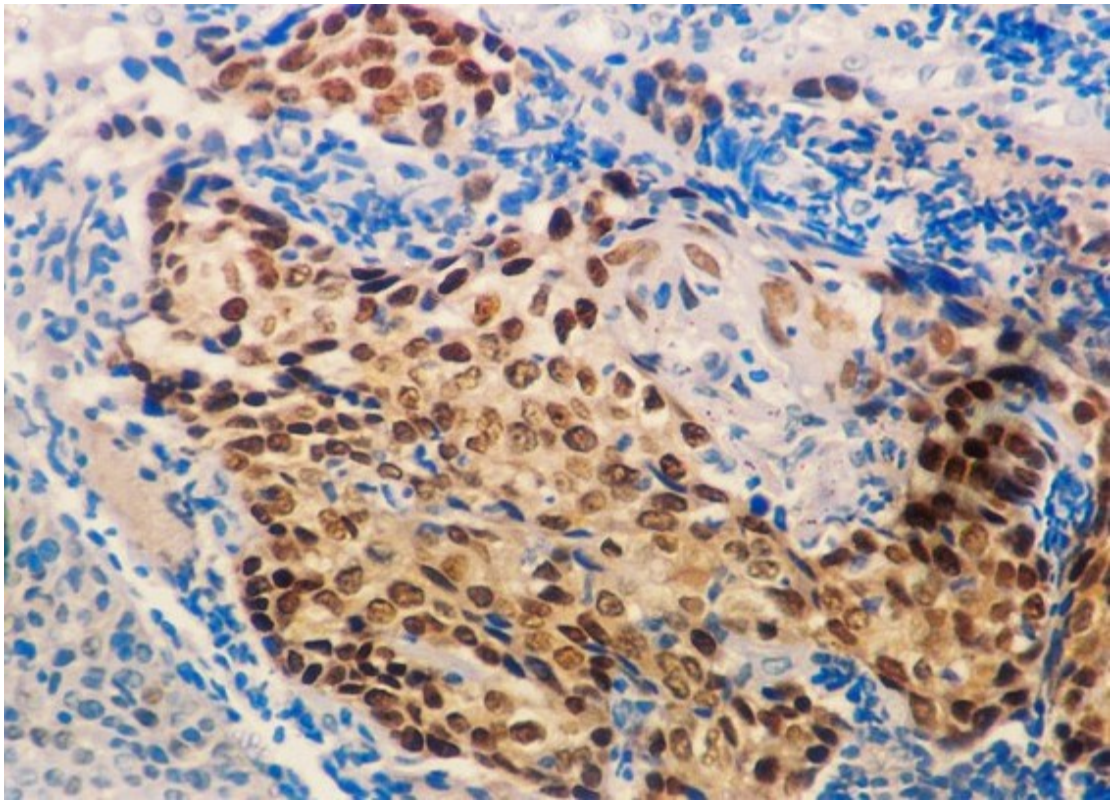

Fig.11 Squamous cell carcinoma of larynx- Cyclin D1 expression, 80 percent

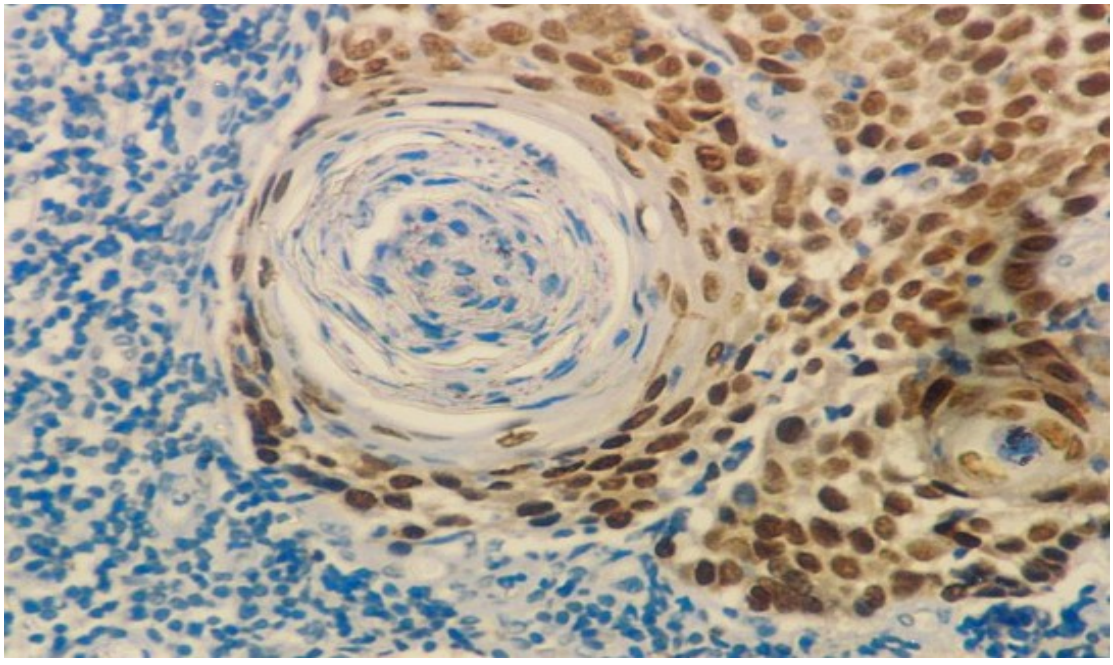

Fig.12 Squamous cell carcinoma of larynx- Cyclin D1 expression, 80 percent

CHAPTER 5

DISCUSSION

The cyclin D1 gene plays a pivotal role in the regulation of the cell cycle[89]. During the middle to late G1phase[90], it complexes with cdk4 or cdk6, thus promoting the phosphorylation of the pRb gene product and, finally, progression to the S phase[91]

Experimental observations indicate that a moderate over expression of Cyclin D 1 leads to a shortening of the G1 phase and less dependence on growth factors for cell proliferation. [102,103] .

It has likewise also been recently reported that antisense to Cyclin D 1 is capable of inhibiting the growth and tumorigenicity of human colon cancer cells, which thus suggests that increased cyclin D1 expression might contribute to the abnormal growth of tumor cells[104]

Cyclin Dl gene aberrations are frequently detectable in head and neck tumors[105-110, 88,111-113] especially in carcinomas of the hypopharynx and, to a lesser extent, in those of the oropharynx and larynx[114-116]

It has been shown that cyclin D1 can become deregulated as a result of various genetic lesions, including chromosomal inversion and translocation in parathyroid adenomas and B-cell non-Hodgkin lymphomas [92, 93] or gene amplification in carcinomas of the breast [94, 95] liver[96] pancreas [97] esophagus [98]anus [98]ovary[99]bladder [100] and lung [101]

A separate prognostic role has been described for Cyclin D1 protein over expression [87] and CCND1 gene amplification [88], with an impact on relapse-free and overall survival in HNSCC. Studies on breast tumors and also on HNSCC have shown that CCND1 amplification, rather than protein over expression, might have prognostic value [119].

Cyclin D1 gene transcriptional activity normally strictly depends on mitogen stimulation, and leads to cell commitment to mitosis through START checkpoint. An early CCND1 over expression is often detectable without evidence of gene amplification; it can be used for molecular epidemiology but it seems to retain a lower prognostic value, if compared with CCND1 amplification, a marker of aggressiveness in LSCC [88].

It has been reported that Cyclin D 1 amplification and/or over expression are associated with unfavorable clinico pathologic features[114, 116] tumor recurrence, and a poor prognosis[111, 115,117,118]

Out of 74 samples studies, Cyclin D 1 protein was over expressed in more than 5% of cells of 23 samples (31.1%) studied, whereas other studies of tumors of the head and neck region (which included only a small number of laryngeal carcinomas) has reported that cyclin D1 was expressed in 35% to 64%[87,111,172,181]

This apparent discrepancy might be explained by the fact that all of these studies included tumors of hypopharynx, in which Cyclin D 1 aberrations have been represent a common event in this regard, it is worth noting that Masuda

et al.,.[185] found cyclin D 1 over expression in 55% of their hypopharyngeal carcinomas.

In this study, Cyclin D 1 over expression was not significantly associated with unfavorable clinico pathologic features such as advanced clinical stage (p=0.3506) and presence of lymphnode metastasis (p=0.5766). This data is in contrast to other studies [87,107,181] where both advanced stage and nodal metastasis were associated with Cyclin D 1 over expression and poor outcome

There was a significant association with Cyclin D 1 expression and extent of tumor (T status) in this study (p=0.0409). This is in line with that of other studies.

Cyclin D 1 expression was not significantly associated with Age of the patient, Grade of the tumor and anatomical subsite of tumor in the larynx.

In contrast to many studies which show Cyclin D 1 over expression and its association with poor outcome in laryngeal cancer[87, 107,172,181] this study did not show any association with time to recurrence of the tumor and time to residual disease after treatment with Radiotherapy.

CONCLUSION

In this study only the Extent of tumor (T status) correlates with the over expression of Cyclin D 1.

Otherwise this study did not support the hypothesis that Cyclin D1 over expression is an independent significant predictor for Stage of disease, Nodal status, Grade of tumor or Recurrence of disease.

Possibly Cyclin D 1 gene amplification may be better prognosticator than it's protein over expression as studied by Bellacosa et.al.[88], Kyomoto et.al, [116].

Distinct molecular pathways at different anatomical regions involved, not only in the abnormal function of the Cyclin D 1 cell cycle regulator, but also in the development of tumors without Cyclin D 1 alteration.[186]

REFERENCE

1. Shah JP, Karnell LH, Hoffman HT, *et al*., Patterns of care for cancer of the larynx in the United States. Arch Otolaryngol – Head Neck Surgery, 123, 475–483, 1997.

2. Jemal A, Tiwari RC, Murray T, *et al*., Cancer statistics, 2004. CA Cancer J Clin, 54, 8–29, 2004.

3. Huang Q, Yu GP, McCormick SA, et al. Genetic differences detected by comparative genomic hybridization in head and neck squamous cell carcinomas from different tumor sites: construction of oncogenetic trees for tumor progression. Genes Chromosomes Cancer, 34, 224–233, 2002.

4. Bosch FX, Ritter D, Enders C, *et al*., Head and neck tumor sites differ in prevalence and spectrum of p53 alterations but these have limited prognostic value. Int J Cancer, 111, 530–538, 2004.

5. Fried MP, Diehl Jr WH, Brownson RJ, *et al*., Cervical metastasis from an unknown primary. Ann Otol Rhinol Laryngol, 84,152–157, 1975.

6. Califano J, Westra WH, Koch W, *et al*., Unknown primary head and neck squamous cell carcinoma: molecular identification of the site of origin. J Natl Cancer Inst, 91,
599– 604, 1999.

7. Spector JG, Sessions DG, Haughey BH, *et al*., Delayed regional metastases, distant metastases, and second primary malignancies in squamous cell carcinomas of the larynx and hypopharynx. Laryngoscope, 111, 1079–1087, 2001.

8. Franchin G, Minatel E, Gobitti C, *et al*., Radiotherapy for patients with early-stage glottic carcinoma: univariate and multivariate analyses in a group of consecutive, unselected patients. Cancer, 98, 765–772, 2003.

9. Reddy SP, Mohideen N, Marra S, *et al*., Effect of tumor bulk on local control and survival of patients with T1 glottic cancer. Radiother Oncol, 47, 161–166, 1998.

10. Rudoltz MS, Benammar A, Mohiuddin M. Prognostic factors for local control and survival in T1 squamous cell carcinoma of the glottis. Int J Radiat Oncol Biol Phys, 26, 767–772, 1993.
11. Licitra L, Bernier J, Grandi C, *et al*., Cancer of the larynx. Crit Rev Oncol Hematol, 47, 65–80, 2003.
12. Almadori G, Galli J, Cadoni G, *et al*., Prospects and therapeutic decisions in the light of biological findings in laryngeal cancer . Acta Otorhinolaryngol Ital, 20, 407–412, 2000.
13. Califano J, van der Riet P, Westra W, *et al*., Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res, 56, 2488–2492, 1996.
14. Uhlman DL, Adams G, Knapp D, *et al*., Immuno histochemical staining for markers of future neoplastic progression in the larynx. Cancer Res, 56, 2199–2205, 1996.
15. Papadimitrakopoulou VA, Liu DD, Mao L, *et al*., Biologic correlates of a biochemoprevention trial in advanced upper aerodigestive tract premalignant lesions. Cancer Epidemiol Biomarkers Prev, 11, 1605–1610, 2002.
16. Califano J, Westra WH, Meininger G, *et al*., Genetic progression and clonal relationship of recurrent premalignant head and neck lesions. Clin Cancer Res, 6, 347–352, 2000.
17. Sanz-Ortega J, Valor C, Saez MC, *et al*., 3p21, 5q21, 9p21 and 17p13 allelic deletions accumulate in the dysplastic spectrum of laryngeal carcinogenesis and precede malignant transformation. Histol Histopathol, 18, 1053–1057, 2003.
18. Shin DM, Kim J, Ro JY, *et al*., Activation of p53 gene expression in premalignant lesions during head and neck tumorigenesis. Cancer Res, 54, 321–326, 1994.
19. Shin DM, Lee JS, Lippman SM, *et al*.,. p53 expressions: predicting recurrence and second primary tumors in head and neck squamous cell carcinoma. J Natl Cancer Inst, 88, 519–529, 1996.

20. Homann N, Nees M, Conradt C, *et al*., Over expression of p53 in tumor-distant epithelia of head and neck cancer patients is associated with an increased incidence of second primary carcinoma. Clin Cancer Res, 7, 290–296, 2001.
21. Carey TE. Field cancerization: are multiple primary cancers monoclonal or polyclonal. Ann Med, 28, 183–188, 1996.
22. Chung KY, Mukhopadhyay T, Kim J, *et al*., Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. Cancer Res, 53, 1676–1683, 1993.
23. Braakhuis BJ, Tabor MP, Kummer JA, *et al*., A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer Res, 63, 1727–1730, 2003.
24. Osman I, Sherman E, Singh B, *et al*., Alteration of p53 pathway in squamous cell carcinoma of the head and neck: impact on treatment outcome in patients treated with larynx preservation intent. J Clin Oncol, 20, 2980–2987, 2002.
25. ScheffnerM, Huibregtse JM, Vierstra RD, *et al*., The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell, 75, 495 505, 1993.
26. Izzo JG, Papadimitrakopoulou VA, Li XQ, *et al*., Dysregulated cyclin D1 expression early in head and neck tumorigenesis: in vivo evidence for an association with subsequent gene amplification. Oncogene, 17, 2313–2322, 1998.
27. Hohaus S, Cavallo S, Bellacosa A, *et al*.,. Telomerase activity in human laryngeal squamous cell carcinomas. Clin Cancer Res, 2, 1895–1900, 1996.
28. Cloos J, Spitz MR, Schantz SP, *et al*., Genetic susceptibility to head and neck squamous cell carcinoma. J Natl Cancer Inst, 88, 530–535, 1996.
29. Rebbeck TR. Molecular epidemiology of the human glutathioneS-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility. Cancer Epidemiol Biomarkers Prev, 6, 733–743, 1997.

30. Lazarus P, Park JY. Metabolizing enzyme genotype and risk for upper aero digestive tract cancer. Oral Oncol, 36, 421–431, 2000.
31. Zheng Z, Park JY, Guillemette C, *et al*., Tobacco carcinogen detoxifying enzyme UGT1A7 and its association with orolaryngeal cancer risk. J Natl Cancer Inst, 93, 1411–1418, 2001.
32. Varzim G, Monteiro E, Silva R, *et al*., Polymorphisms of arylamine N-acetyl transferase (NAT1 and NAT2) and larynx cancer susceptibility. ORL J Otorhinolaryngol Relat Spec, 64, 206–212, 2002.
33. Elahi A, Zheng Z, Park J, *et al*., The human OGG1 DNA repair enzyme and its association with orolaryngeal cancer risk. Carcinogenesis, 23, 1229–1234, 2002.
34. Varzim G, Monteiro E, Silva RA, *et al*., CYP1A1 and XRCC1 gene polymorphisms in SCC of the larynx. Eur J Cancer Prev, 12, 495–499, 2003.
35. Geisler SA, Olshan AF. GSTM1, GSTT1, and the risk of squamous cell carcinoma of the head and neck: a mini-HuGE review. Am J Epidemiol, 154, 95–105, 2001.
36. Jourenkova-Mironova N, Voho A, Bouchardy C, *et al*., Glutathione S-transferase GSTM3 and GSTP1 genotypes and larynx cancer risk. Cancer Epidemiol Biomarkers Prev, 8, 185–188, 1999.
37. Mork J, Lie AK, Glattre E, *et al*., Human papilloma virus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med, 344, 1125–1131, 2001.
38. Gillison ML, Koch WM, Capone RB, *et al*., Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst, 92, 709–720, 2000.
39. Schwartz SM, Daling JR, Doody DR, et al. Oral cancer risk in relation to sexual history and evidence of human papilloma virus infection. J Natl Cancer Inst, 90, 1626–1636, 1998.

40. Almadori G, Cadoni G, Cattani P, *et al*., Detection of human papilloma virus DNA in laryngeal squamous cell carcinoma by polymerase chain reaction. Eur J Cancer, 32A, 783–788, 1996.
41. Kaya H, Kotiloglu E, Inanli S, *et al*., Prevalence of human papilloma virus (HPV) DNA in larynx and lung carcinomas. Pathologica, 93, 531–534, 2001.
42. Jacob SE, Sreevidya S, Chacko E, *et al*., Cellular manifestations of human papillomavirus infection in laryngeal tissues. J Surg Oncol, 79, 142–150, 2002.
43. Venuti A, Manni V, Morello R, *et al*., Physical state and expression of human papilloma virus in laryngeal carcinoma and surrounding normal mucosa. J Med Virol, 60, 396–402, 2000.
44. Atula S, Grenman R, Kujari H, *et al*., Detection of human papilloma virus (HPV) in laryngeal carcinoma cell lines provides evidence for a heterogeneic cell population. Eur J Cancer, 35, 825–832, 1999.
45. Azzimonti B, Hertel L, Aluf. P, *et al*., Demonstration of multiple HPV types in laryngeal premalignant lesions using polymerase chain reaction and immuno histochemistry. J Med Virol, 59, 110–116, 1999.
46. Lindeberg H, Krogdahl A. Laryngeal cancer and human papilloma virus: HPV is absent in the majority of laryngeal carcinomas. Cancer Lett, 146, 9–13, 1999.
47. Ha PK, Pai SI, Westra WH, *et al*., Real-time quantitative PCR demonstrates low prevalence of human papilloma virus type 16 in premalignant and malignant lesions of the oral cavity. Clin Cancer Res, 8, 1203–1209, 2002.
48. Garrett LR, Perez-Reyes N, Smith PP, *et al*., Interaction of HPV-18 and nitrosomethylurea in the induction of squamous cell carcinoma. Carcinogenesis, 14, 329–332, 1993.
49. Park NH, Gujuluva CN, Baek JH, *et al*., Combined oral carcinogenicity of HPV-16 and benzo(a)pyrene: an in vitro multistep carcinogenesis model. Oncogene, 10, 2145–2153, 1995.

50. Liu X, Han S, Baluda MA, *et al*., HPV-16 oncogenes E6 and E7 are mutagenic in normal human oral keratinocytes. Oncogene, 14, 2347–2353, 1997.
51. Almadori G, Galli J, Cadoni G, *et al*., Human papilloma virus infection and cyclin D1 gene amplification in laryngeal squamous cell carcinoma: biologic function and clinical significance. Head Neck, 24, 597–604, 2002.
52. Cattani P, Hohaus S, Bellacosa A, et al. Association between cyclin D1 (CCND1) gene amplification and human papillomavirus infection in human laryngeal squamous cell carcinoma. Clin Cancer Res, 4, 2585–2589, 1998.
53. Almadori G, Cadoni G, Cattani P, *et al*., Human papilloma virus infection and epidermal growth factor receptor expression in primary laryngeal squamous cell carcinoma. Clin Cancer Res, 7, 3988–3993, 2001.
54. Capone RB, Pai SI, Koch WM, *et al*., Detection and quantization of human papilloma virus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. Clin Cancer Res, 6, 4171–4175, 2000.
55. Sporn MB. Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer Res, 36, 2699–2702, 1976.
56. Johnson FL. Management of advanced premalignant laryngeal lesions. Curr Opin Otolaryngol Head Neck Surg, 11, 462–466, 2003.
57. Papadimitrakopoulou VA, Clayman GL, Shin DM, et al. Bio chemoprevention for dysplastic lesions of the upper aerodigestive tract. Arch Otolaryngol Head Neck Surg, 125, 1083–1089, 1999.
58. van Zandwijk N, Dalesio O, Pastorino U, et al. EUROSCAN, a randomized trial of vitamin A and N-acetyl cysteine in patients with head and neck cancer or lung cancer. For the European Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperative Groups. J Natl Cancer Inst;92:977–986, 2000.
59. Steinbach G, Lynch PM, Phillips RK, *et al*., The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med, 342, 1946–1952, 2000.

60. Lin DT, Subbaramaiah K, Shah JP, *et al*., Cyclooxygenase-2: a novel molecular target for the prevention and treatment of head and neck cancer. Head Neck, 24, 792–799, 2002.
61. Ranelletti FO, Almadori G, Rocca B, *et al*., Prognostic significance of cyclooxygenase-2 in laryngeal squamous cell carcinoma. Int J Cancer, 95, 343–349, 2001.
62. Almadori G, Bussu F, Galli J, *et al*., Serum folate and homocysteine levels in head and neck squamous cell carcinoma. Cancer, 94, 1006–1011, 2002.
63. Almadori G, Bussu F, Galli J, *et al*., Serum Levels of Folate, Homocysteine And Vitamin B12 In Head And Neck Squamous Cell Carcinoma And In Laryngeal Leucoplakia. Cancer, in press.. Ref Type: Generic, 2004.
64. Kato I, Dnistrian AM, Schwartz M, *et al*., Serum folate, homocysteine and colorectal cancer risk in women: a nested case-control study. Br J Cancer 1999, 79, 1917–1922.
65. Heimburger DC, Alexander CB, Birch R, *et al*., Improvement in bronchial squamous metaplasia in smokers treated with folate and vitamin B12. Report of a preliminary randomized, doubleblind intervention trial. JAMA, 259, 1525–1530, 1988.
66. Saito M, Kato H, Tsuchida T, *et al*., Chemoprevention effects on bronchial squamous metaplasia by folate and vitamin B12 in heavy smokers. Chest, 106, 496–499, 1994.
67. Xiao SD, Meng XJ, Shi Y, *et al*., Interventional study of high dose folic acid in gastric carcinogenesis in beagles. Gut, 50, 61–64, 2002.
68. Nathan CA, Sanders K, Abreo FW, *et al*., Correlation of p53 and the proto-oncogene eIF4E in larynx cancers: prognostic implications. Cancer Res, 60, 3599–3604, 2000.
69. Reed AL, Califano J, Cairns P, *et al*., High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res, 56, 3630–3633, 1996.

70. Smigiel R, Sasiadek M, Krecicki T, *et al*., Inactivation of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene in squamous cell carcinoma of the larynx. Mol Carcinog, 39, 147–154, 2004.
71. Sasiadek MM, Stembalska-Kozlowska A, Smigiel R, *et al*., Impairment of MLH1 and CDKN2A in oncogenesis of laryngeal cancer. Br J Cancer, 90, 1594–1599, 2004.
72. Brennan JA, Mao L, Hruban RH, *et al*., Molecular assessment of histo pathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med, 332, 429–435, 1995.
73. Belbin TJ, Singh B, Barber I, *et al*., Molecular classification of head and neck squamous cell carcinoma using cDNA microarrays. Cancer Res, 62, 1184–1190, 2002.
74. Leethanakul C, Patel V, Gillespie J, *et al*., Gene expression profiles in squamous cell carcinomas of the oral cavity: use of laser capture micro dissection for the construction and analysis of stage-specific cDNA libraries. Oral Oncol, 36, 474–483, 2000.
75. Maurizi M, Scambia G, Benedetti Panici P, *et al*., EGF receptor expression in primary laryngeal cancer: correlation with clinico pathological features and prognostic significance. Int J Cancer 2, 52, 862–866, 1994.
76. Maurizi M, Almadori G, Ferrandina G, *et al*., Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma. Br J Cancer, 74, 1253–1257, 1996.
77. Almadori G, Cadoni G, Galli J, *et al*., Epidermal growth factor receptor expression in primary laryngeal cancer: an independent prognostic factor of neck node relapse. Int J Cancer, 84, 188–191, 1999.
78. Rubin Grandis J, Melhem MF, Gooding WE, *et al*., Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. J Natl Cancer Inst, 90,824–832m, 1998.

79. Wen QH, Miwa T, Yoshizaki T, *et al*., Prognostic value of EGFR and TGF-alpha in early laryngeal cancer treated with radiotherapy. Laryngoscope, 106, 884–888, 1993.
80. Dassonville O, Formento JL, Francoual M, *et al*., Expression of epidermal growth factor receptor and survival in upper aero digestive tract cancer. J Clin Oncol, 11, 1873–1878, 1993.
81. Gupta AK, McKenna WG, Weber CN, *et al*., Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. Clin Cancer Res, 8, 885–892, 2002.
82. Narayana A, Vaughan AT, Gunaratne S,. *et al*., Is p53 an independent prognostic factor in patients with laryngeal carcinoma . Cancer, 82, 286–291, 1998.
83. Alsner J, Sorensen SB, Overgaard J. TP53 mutation is related to poor prognosis after radiotherapy, but not surgery, in squamous cell carcinoma of the head and neck. Radiother Oncol, 59, 179–185, 2001.
84. Anwar K, Nakakuki K, Imai H, *et al*., Over-expression of p53 protein in human laryngeal carcinoma. Int J Cancer 1993, 53, 952–956.
85. Maestro R, Dolcetti R, Gasparotto D, *et al*., High frequency of p53 gene alterations associated with protein over expression in human squamous cell carcinoma of the larynx. Oncogene, 7, 1159–1166, 1992.
86. Taylor D, Koch WM, Zahurak M, *et al*., Immuno histochemical detection of p53 protein accumulation in head and neck cancer: correlation with p53 gene alterations. Hum Pathol, 30, 1221–1225, 1999.
87. Pignataro L, Pruneri G, Carboni N, *et al*., Clinical relevance of cyclin D1 protein overexpression in laryngeal squamous cell carcinoma. J Clin Oncol 1998, 16, 3069–3077.
88. Bellacosa A, Almadori G, Cavallo S, *et al*., Cyclin D1 gene amplification in human laryngeal squamous cell carcinomas: prognostic significance and clinical implications. Clin Cancer Res 1996, 2, 175–180.

89. Sherr CJ: Mammalian GI cyclins. Cell 73:1059-1065, 1993
90. Baldin V, Lukas J, Marcote MJ, *et al*., Cyclin D1 is a nuclear protein required for cell cycle progression in GI. Genes Dev 7:812-821, 1993
91. Sherr CJ: G1 phase progression: Cycling on cue. Cell 79:551-555, 1994
92. Motokura T, Bloom T, Kim HG, *et al*., A novel cyclin encoded by a bcl ll-linked candidate oncogene. Nature 350:512-515, 1991
93. Rosemberg CL, Wong E, Petty EM, *et al*., PRAD1, a candidate BCL1 oncogene: Mapping and expression in centrocytic lymphoma. Proc Natl Acad Sci USA 88:9638-9642, 1991
94. Lammie GA, Fantl V, Smith R, *et al*., DllS287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to *bcl*- 1. Oncogene 8:439-444, 1991
95. Gillett C, Fantl V, Smith R, *et al*., Amplification and over expression of cyclin Dl in breast cancer detected by immuno histochemical staining. Cancer Res 54:1812-1817, 1994
96. Zhang Y-J, Jiang W, Chen CJ, *et al*., Amplification and over expression of cyclin D1 in human hepato cellular carcinoma. Biochem Biophys Res Commun 196:1010-1016, 1993
97. Gansauge S, Gansauge F, Ramadani N, *et al*., over expression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis. Cancer Res 57:1634-1637, 1997
98. Sheyn I, Noffsinger AE, Heffelfinger S, et al: Amplification and expression of the cyclin D1 gene in anal and esophageal squamous cell carcinomas. Hum Pathol 28:270-276, 1997
99. Courjal F, Louason G, Speiser P, *et al*., Cyclin gene amplification and over expression in breast and ovarian cancers: Evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. Int J Cancer 69:247-253, 1996

100. Bringuier PP, Tamimi Y, Schuuring E, *et al*., Expression of cyclin D1 and EMS1 in bladder tumours; Relationship with chromosome 11q13 amplification. Oncogene 12:1747-1753, 1996
101. Betticher DC, Heighway J, Hasleton PS, *et al*., Prognostic significance of CCNDI (cyclin D1) over expression in primary resected non-small-cell lung cancer. Br J Cancer 73:294-300, 1996
102. Quelle DE, Ashmun RA, Shurtleff SA, *et al*.,: Over expression of mouse D-type cyclins accelerates **GI** phase in rodent fibroblasts. Genes Dev 7:1559-1571, 1993
103. Zwijsen RM, Klompmaker R, Wientjens EB, *et al*., Cyclin D1 triggers autonomous growth of breast cancer cells by governing cell cycle exit. Mol Cell Biol 16:2554-2560, 1996
104. Arber N, Doki Y, Han EK, *et al*., Antisense to cyclin DI inhibits the growth and tumorigenicity of human colon cancer cells. Cancer Res 57:1569-1574, 1997
105. Berenson JR, Yang J, Mickel RA: Frequent amplification of *bcl*- 1 locus in head and neck carcinomas. Oncogene 4:1111-1116, 1989
106. Jares P, Fernandez PL, Campo E, *et al*., PRAD-1/cyclin DI gene amplification correlates with messanger mRNA over expression and tumor progression in human laryngeal carcinoma. Cancer Res 54: 4813-7 1994
107. Bartkova J, Lukas J, Muller H, *et al*., Abnormal patterns of D-type cyclin expression and G1 regulation in human head and neck cancer. Cancer Res 55:949-956, 1995
108. Fracchiolla NS, Pignataro L, Capaccio P. *et al*., Multiple genetic lesions in laryngeal squamous cell carcinomas. Cancer 75:1292-1301, 1995
109. Gaffey MJ, lezzoni JC, Meredith SD, *et al*., Cyclin D1 (PRAD1, CCND1) and glutathione-S-transferase pi gene expression in head and neck squamous cell carcinoma. Hum Pathol 26:1221-1226, 1995

110. Davidson BJ, Lydiatt WM, Abate MP, *et al*., Cyclin D1 abnormalities and tobacco exposure in head and neck squamous cell carcinoma. Head Neck 18:512-521, 1996
111. Akervall JA, Michalides RJ AM, Mineta H, *et al*., Amplification of cyclin D1 in squamous cell carcinoma of the head and neck and the prognostic value of chromosomal abnormalities and cyclin DI over expression. Cancer 79:380-389, 1997
112. Fortin A, Guerry M, Guerry R, *et al*., Chromosome 11q13 gene amplifications in oral and oropharyngeal carcinomas: No correlation with sub clinical lymph node invasion and disease recurrence. Clin Cancer Res 3:1609-1614, 1997
113. Mineta H, Borg A, Dictor M, *et al*., Correlation between p53 mutation and cyclin D 1 amplification in head and neck squamous cell carcinoma. Oral Oncol 33:42-46, 1997
114. Muller D, Millon R, Lidereau R, *et al*., Frequent amplification of 11ql3 DNA markers is associated with lymph node involvement in human head and neck squamous cell carcinomas. Oral Oncol Eur J Cancer 30:113-120, 1994
115. Michalides RJ AM, van Veelen N, Hart A, *et al*., over expression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck. Cancer Res 55:975-978, 1995
116. Kyomoto R, Kumazawa H, Toda Y, *et al*., Cyclin-D1-gene amplification is a more potent prognostic factor than its protein over-expression in human head-and-neck squamous-cell carcinoma. Int J Cancer 74:576-581, 1997
117. Meredith SD, Levine PA, Burns JA, *et al*., Chromosome 11q13 amplification in head and neck squamous cell carcinoma. Association with poor prognosis. Arch Otolaryngol Head Neck Surg 121:790-794, 1995
118. Michalides RJ AM, van Veelen NMJ, Kristel PMP, *et al*., Over expression of cyclin D1 indicates a poor prognosis in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 123:497-502, 1997.

119. Maurizi M, Almadori G, Cadoni G, et al. Cathepsin D concentration in primary laryngeal cancer: correlation with clinico-pathological parameters, EGFR status and prognosis. Int J Cancer, 69, 105–109, 1996.
120. Piantelli M, Iacobelli S, Almadori G, *et al*., Lack of expression of galectin-3 is associated with a poor outcome in node-negative patients with laryngeal squamous-cell carcinoma. J Clin Oncol, 20, 3850–3856, 2002.
121. Lauriola L, Michetti F, Maggiano N, et al. Prognostic significance of the Ca(2+) binding protein S100A2 in laryngeal squamous-cell carcinoma. Int J Cancer, 89, 345–349, 1998.
122. Ferrandina G, Almadori G, Maggiano N, *et al*., Growth inhibitory effect of tamoxifen and quercetin and presence of type II estrogen binding sites in human laryngeal cancer cell lines and primary laryngeal tumors. Int J Cancer, 77, 747–754, 1998.
123. Maurizi M, Ferrandina G, Almadori G, *et al*., Prognostic significance of methyl-p-hydroxy-phenyllactate-esterase activity in laryngeal squamous cell carcinoma. Br J Cancer, 77,1253–1259, 1998.
124. Cancer of the larynx, MMTR, Chennai 1998-2002.
125. Stephenson VT, Barnes DE, Holmes FF, *et al*., Gender influences sub site of origin of laryngeal carcinoma. Arch otolaryngol Head and Neck Surg 117: 774; 1991. 126. Cancer of the larynx, Chennai HCR, 1998-2002.
127. Austin DF; Larynx. In schottenfield D, Frauman JF (eds): Cancer Epidemiology and prevention, Philadelphia, WB Saunders 1982.
128. Simon M, Khan T, Schneider A, *et al*., laryngeal carcinoma in a 12 year old child. Arch otolaryngol Head and Neck Surg 120: 277, 1994.
129. Boring CC, Saniers TS, Tong T, *et al*., Cancer statistics, 1994 CA 44: 7, 1994.
130. Ferlay J, Bran F, Piscni P. Cancer incidence, mortality and prevalence worldwide, version 1.0, Lyon: IARC press, IARC cancer base No. 5; 2001.

131. ENCR. European Network of Cancer Registries. European Cancer Incidence and mortality (EUROCIM). Lyon: International Agency for Research on Cancer, 1997.

132. Shah JP, Karnell LH, Hoffman VT, *et al*., Patterns of care for cancer of the larynx in the United States. Arch otolaryngol Head and Neck Surgery 123: 475-483, 1997.

133. Falli R, Pickle LW, Brown LM, Effects of smoking and alcohol consumption on laryngeal cancer risk in coastal Texas, Cancer Res 49: 4024, 1989.

134. Brugers J, Guenel P, Lecelerc A *et al*., Differentail effects of tobacco and alcohol in cancer of larynx, pharynx and mouth. Cancer 57: 391, 1986.

135. Worlley P, Vaghan TL, Dais S, *et al*., A case control study of occupational risk factors for laryngeal cancer. Br. J Ind Med 49: 837, 1982.

136. Brown LM, Mason TJ, Pickle LW *et al*., occupational risk factors for laryngeal cance on the Texas gulf coast: Cancer Res 48: 1960, 1988.

137. Amendola BE, Amendola MA, Mc Clarkey KD. Radiation induced carcinoma of larynx. Surg Gynecol Obstet 161: 30, 1985 .

138.Zheng W, Blot WJ Shu XO *et al*., Diet and other risk factors for laryngeal cancers in Shangai, China. Am J Epidemiol 136: 128, 1992 .

139. Freudenheim JL, Graham S Byers TE, *et al*., Diet, smoking and alcohol in cancer of the larynx. A case- control study. Nutr. Cancer 17: 33, 1992.

140. Shin DM, Lee JS, Lippman SM, *et al*., p53 expression: predicting recurrence and second primary tumor in head and neck squamous cell carcinoma. J. Natl. Cancer Inst 88,519-529, 1996.

141. Bosch FX, Ritter D, Enders C *et al*., Head and neck differ in prevalence and spectrum of p53 alterations but these have limited prognostic value. Int J Cancer 111, 530-538, 2004.

142. Cancer of the Head and Neck Chapter 20; cancer of larynx, Robert J, Sinard, Netter ville *et al*., 2004.

143. Shah JP, cervical nodal metastasis-diagnostic, therapeutic and prognostic implications. Oncology, 4:61, 1990.

144. Harabayashi H, Koshii K Uno K, *et al*., Extra capsular spread of SCC in neck lymphnodes: prognostic factors of laryngeal cancer. Laryngoscope 101: 502, 1993

145. Jones AS, Rokand NJ, Field JK, *et al*.,The level of cervical lymphnode metastasis-Their prognostic relevance and relationshiop with head and neck squamous primary sites. Clin otolaryngol, 19: 63, 1994.

146. Esteban F, Moreno JA, Delgedo, Rodriguez M, *et al*., Risk factors involved in stomal recurrence following laryngectomy. J laryngolo otol, 107: 527, 1993.

147. Manni JJ, Terharard CH, de Boer MJ, *et al*., Prognostic factors for survival in patients with T 3 laryngeal carcinoma. Am J Surg, 164: 682; 1992.

148. Goldsmith MM, Pillsbury HC, Pathologic and cytometric indicators of biologic behaviour in laryngeal carcinoma. In Silver CE(ed): laryngeal cancer , Thieme, New york,1991.

149. Rudoltz MS, Benammar A, Mohiuddi M . Prognostic factors for local control and survival in T 1 Squamous cell carcinoma of the glottis. Int J Radiat Oncol Biol Phys , 26, 767-772, 1993.

150. Evans GI, Vonsden KH, Proliferation, cell cycle and apoptosis in cancer. Nature 411: 342-8, 2001.

151. Hartwell LH, Weinert TA,. Checkpoints: controls that ensure the order of cell cycle events. Science 246: 629-34, 1989.

152. Nigg EA. Mitotic kinases as regulators of cell division and its check points. Nature Rev Mol Cell Bio, 2: 21-32; 2001.

153. Cahill DP, YuJ et.al. Mutations of mitotic check point genes in human cancer. Nature 392: 300-3; 1998.

154. Harper JW, Adams PD. Cyclin dependent kinases. Chem Rev 101: 2511-26; 2001.

155. Obaya AJ, Sedivy JM. Regulation of cyclin, cdk activity in mammalian cells. Cell mol life sci , 59: 126-42; 2002.
156. Matsushime H, Sherr CJ, Ashmun RA, colony stimulating factor 1 regulates novel cyclins during the G 1 phase of the cell cycle. Cell 65: 701-13, 1991
157. Goodrich DW, Wang NP et al. The retinoblastoma gene product regulates progression through the G 1 phase of the cell cycle. Cell 67: 293-302, 1991
158. Connel- Crowley L, Harper Jw, Goodrich DW. Cyclin D 1/ cdk 4 regulates retinoblastoma protein mediated cell cycle arrest by site specific phosphorylation. Mol Biol cell 8: 287-391, 1997
159. Lee WH. The molecular basis of cancer suppression by the retinoblastoma gene. International symposiym of the Princess Takamatsu Cancer Research Fund . 20:159-70, 1989
160. Malumbres M, Barbacid M. To cycle or not to cycle: A critical decision in cancer. Nature reviews 1: 222-231, 2001
161. Hall M , Peters G. Genetic alterations of cyclins, cyclin dependent kinases and cdk inhibitors in human cancer. Adv cancer Res 68:67-108,1996
162. Hunt T. Cyclins and their partner: from a single idea to complicated reality, Semin. Cell Biol 2: 213-219, 1991
163. Glotzer M, Murrary AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. Nature 349: 132-4 , 1991.
164. Hunt T, Luca FC , Ruderman JV. The requirements for protein synthesis and degradation and control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. J Cell boil, 116: 707-11,. 1992.165. Rechsteiner M, Rogers SW. PEST sequences and regulation by proteolysis. TIBS, 21: 267-271; 1996.166. Morgan DO, Cyclin dependent kinases: Engines, clocks and microprocessors. Annu.Rev. Cell Dev. Biol., 13:261-91; 1997.

167. Serrano M, Hannon GJ and Beach D. A new regulatory motif in cell control causing specific inhibition of cyclin D /CDK 4. Nature, 366: 704-707, 1993.

168. Inaba T, Matsushime H, Valentune M et al., Genomic Organization, chromosomal localization and independent expression of human Cyclin D genes. Genomics, 13: 565-574, 1992.

169. Dowdy SF, Hinds PW, Louie K *et al*.,Physical Interaction of the retinoblastoma protein with human D cyclins . Cell, 73: 499-511, 1993.

170. Kinoshita I, Akita DH, Mishina Takayuki *et al*., Altered p16 INK 4 and retinoblastoma protein status in non small cell lung cancer: Potential synergestic effect with altered p53 protein on proliferative activity. Cancer Res. 56: 5557-62, 1996.

171. Vielba R, Bilbao J, Ispizua *et al*., p53 and Cyclin D1 as prognostic factors in squamous cell carcinoma of the larynx**.** Laryngoscope. Jan; 113(1):167-72, 2003.

172. Michalides RJ, van Veelen NM, Kristel PM *et al*., over expression of Cyclin D1 indicates a poor prognosis in squamous cell carcinoma of the head and neck Arch Otolaryngol Head Neck Surg. May; 123(5):497-502, 1997.

173**.**Georgiou A, Gomatos IP, Ferekidis E, Syrigos *et al*., Prognostic significance of p53, bax and bcl-2 gene expression in patients with laryngeal carcinoma **,**Eur J Surg Oncol. Sep; 27(6):574-80, 2001.

174. Jackel MC, Sellmann L, Youssef S, Dorudian MA, Fuzesi L. *et al*., Prognostic significance of expression of p53, bcl-2 and bax in squamous epithelial carcinoma of the larynx--a multivariate analysis : HNO. Mar; 49(3):204-11, 2001.

175. Mielcarek-Kuchta D, Olofsson J, Golusinski W. p53, Ki67 and Cyclin D1 as prognosticators of lymph node metastases in laryngeal carcinoma, Eur Arch Otorhinolaryngol. Nov; 260(10):549-54,2003.

176. Capaccio P, Pruneri G, Carboni N, Cesana BM, Pignataro L. Cyclin D1 expression is predictive of occult metastases in head and neck cancer patients with clinically negative cervical lymph nodes**,** Head Neck. May; 22(3):234-40, 2000.

177. Liu SC, Zhang SY, Babb JS, Ridge JA, Klein-zantoAJ. Image cytometry of Cyclin D1: a prognostic marker for head and neck squamous cell carcinomas[177]Cancer Epidemiol Biomarkers Prev. May; 10(5):455-9, 2001.

178.Namazie A, Alavi S, Olopade OI, Pauletti G, *et al.*, Cyclin D1 amplification and p16 (MTS1/CDK4I) deletion correlates with poor prognosis in head and neck tumors, Laryngoscope. Mar; 112(3):472-81, 2002.

179. Rodrigo Tapia JP, Gonzalez Meana MV, *et al.*, Inactivation of p53 and amplification of cyclin D1 in squamous cell carcinomas of the head and neck, Acta Otorrinolaringol Esp. Apr; 53(4):225-32, 2002

180.Ioachim E, Peschos D, Goussia A, Mittari E, Expression patterns of Cyclins D1, E in laryngeal epithelial lesions: correlation with other cell cycle regulators (p53, pRb, Ki-67 and PCNA) and clinico pathological features ,J Exp Clin Cancer Res. Jun;23(2):277-83, 2004

181.Michalides R, van Veelen N, Hart A, *et al.*, Over expression of Cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck, Cancer Res. Mar 1; 55(5):975-8, 1995

182. Fracchiolla NS, Pruneri G, Pignataro L *et al.*, Molecular and immuno histochemical analysis of the bcl-1/cyclin D1 gene in laryngeal squamous cell carcinomas: correlation of protein expression with lymph node metastases and advanced clinical stage ,Cancer. Mar 15; 79(6):1114-21, 1997

183. Jares P, Fernandez PL, Campo E, Nadal A, *et al.*, PRAD-1/cyclin D1 gene amplification correlates with messenger RNA over expression and tumor progression in human laryngeal carcinomas, Cancer Res. Sep 1;54(17):4813-7, 1994

184. Wang MB, Yip HT, Srivatsan ES: Antisense Cyclin D1 enhances sensitivity of head and neck cancer cells to cisplatin, Laryngoscope. Jun; 111(6):982-8 ,2001

185. Masuda M, Hikkawa N, Nakshima T *et al.*, Cyclin D over expression in primary hypopharyngeal carcinomas. Cancer 3: 390-5, 1996.

186. Eurico Monteiro, Graca Varzim, Ana M Pires *et al.*, Cyclin D 1 A 870 G polymorphism and amplification in laryngeal squamous cell carcinoma: implications of tumor localization and tobacco exposure. Cancer Detection and Prevention 28, 237-243, 2004.